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BIOPRODUCTS FOR CONTROL OF WEEDS AND PLANT PATHOGENS

Final Research Report

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LIST OF ABBREVIATIONS

Ala	alanine
$[\alpha]_D$	optical rotation
Asp	asparagine
br	broad
calcd.	calculated
^{13}C NMR	carbon-13 nuclear magnetic resonance
COSY	COrrelated SpectroscopY
δ	chemical shift (ppm)
d	doublet
FA	fatty acid
FAB	fast atom bombardment
FCC	flash column chromatography
GC	gas chromatography
Glu	glutamic acid
h	hour
^1H NMR	proton nuclear magnetic resonance
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum coherence
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
Hz	hertz
Ile	isoleucine
J	coupling constant
Leu	leucine
Lys	lysine
m	multiplet
m / z	mass / charge ratio
MHz	megahertz
min	minute
MS	mass spectrometry
MWCO	molecular weight cut off
Orn	ornithine
PAF	Pseudomonas Agar F
r_t	retention time
Ser	serine
s	singlet
TLC	thin layer chromatography
Thr	threonine
t	triplet
TSA	trypticase soy agar
UV	ultraviolet

a) ABSTRACT/SUMMARY

A sustainable agriculture requires the development of approaches to solve environmental problems posed by the use of pesticides. Biocontrol agents constitute a viable and extremely desirable alternative to the use of chemical pesticides, thus their technical and commercial development is essential.

The goals of this project were to discover bioproducts produced by bacterial biocontrol agents active against plant pathogens and weeds, and to apply this knowledge to optimize production of these products. Our investigation of two potential bacterial biocontrol agents has demonstrated that these agents produce a range of bioproducts active against some of the most important pathogens of canola, such as blackleg, blackspot, stem rot, and root rot fungi, and a mixture of macromolecular metabolites active against a major weed pest, green foxtail. Some of these natural products were isolated as complex mixtures of cyclic peptides, quite hydrophobic and with molecular weight ca. 1,200 Da. The chemical structures of two of these antifungal peptides were established by X-ray crystallography and spectroscopy. Another metabolite belonged to a new type of siderophore, bioproducts well-known as peptidic ion chelators containing non-protein amino acids. Herbicidal components present in bacterial cultures were of peptidic nature, water soluble, and of molecular weight between 10 -30 K.

b) EXECUTIVE SUMMARY

Recent surveys in Saskatchewan for herbicide-resistant wild oats and green foxtail have indicated that out of 201 field sites surveyed, 48% had "group-1" herbicide resistant wild oat populations and 28% had either "group-1" or "group-3" herbicide-resistant green foxtail populations (Beckie, H.J., A. Legere, A.G. Thomas, L.T. Juras, and M.D. Devine; 1996 Survey of Herbicide-Resistant Wild Oat and Green Foxtail in Saskatchewan: Interim Report. AAFC Report, 22 pp.). It was concluded that continued use of these herbicides may lead to significant increases in occurrence of herbicide resistance, particularly group-1 (ACCase) herbicides.

AAFC reports (S. M. Boyetchko) indicate that potential biocontrol agents exhibit an ability to control herbicide-resistant wild oats and green foxtail. These results strongly suggest that these new agents produce "natural products" that may be exploited for control of herbicide-resistant weed populations. In fact, the production of natural products with antimicrobial activity, i.e. antibiotics, appears to be a frequent mechanism utilized by biocontrol agents. These natural products can inhibit microbial and weed growth and therefore control plant pathogens and weeds.

Clear correlations have been established between "antibiotic" production and disease suppression. However, there is no clear correlation between the biocontrol species and the particular herbicidal or antifungal product(s) produced. This implies that a detailed chemical analysis employing modern spectroscopic techniques must be carried out in order to establish the chemical structures of the bioactive natural products of any putative biocontrol agent. Knowledge of the bioactive secondary metabolites produced by biocontrol agents is extremely important for several reasons: 1) the bacterial isolates may be formulated with putative precursors of these bioactive metabolites to increase the biocidal activity of the agent; 2) it may also be possible to apply the novel bioactive natural products as biorational pest control products; or 3) it may be possible to manipulate their biosynthesis to produce more potent biocontrol agents and/or metabolites with higher bioactivity.

In this project, two different bacterial strains (BRG100 and 189) of potential biocontrol agents were studied; the bacterial cells were cultured under different conditions (liquid and solid media of different composition, temperature, incubation period) and the cell-free supernatant of each fermentation was extracted with organic solvents. The chemical analyses of extracts indicated that strains BRG100 and 189 produced different UV-active metabolites in a consistent pattern. The protocol for production and extraction of antifungal components was optimized to improve product yields and minimize culture handling.

Fractionation of bioactive ethyl acetate extracts obtained from strain BRG100, followed by bioassays to determine herbicidal and antifungal activity of those fractions indicated that several bioactive products are produced by strain BRG100. These purified fractions showed strong antifungal activity against important canola, rapeseed and mustards pathogens. Importantly, our chemical analyses of these bioactive fractions, in particular HPLC and NMR spectroscopy, showed that the active products are a complex mixture of cyclic peptides. X-ray crystallography allowed the determination of the chemical structure of two of the antifungal peptides which we named **pseudophomin A** and

pseudophomin B; these are cyclic peptides with nine amino acid residues and lipophilic portion. Pseudophomins **A** and **B** along with corresponding controls were tested against four plant pathogens; **B** showed substantial activity (65%, 51% and 60% at a concentration of 0.5 mg/mL) against *P. lingam*, *A. brassicae* and *S. sclerotiorum*, respectively, and 18% inhibition against *R. solani*, whereas **A** displayed lower antifungal activity 31%, 23% and 13%, and 19% at similar concentration. However compounds **A** and **B** isolated from strain 189 were found inactive against *P. lingam*. Pseudophomins A and B showed slight inhibition (<15%) of root germination and root growth of the weed green foxtail utilizing methods B and C; however, stronger herbicidal activity was detected using method D. interestingly pseudophomin A showed stronger inhibition (67%) of green foxtail root germination than pseudophomin B (30%) at a conc. 5×10^{-4} M. Overall our results indicate that the antifungal activity shown by cultures of *P. fluorescens* strain BRG100 is likely due to the secretion of pseudophomins A (**1**) and B (**2**). Furthermore, considering that strong herbicidal activity remained in the aqueous phase of extracted cultures, pseudophomins do not appear to be the main components responsible for the herbicidal activity shown by BRG100 liquid cultures. Compounds **A** and **B** isolated from strain 189 were found inactive against green foxtail.

We established methods for larger scale separation of the bioactive components produced by bacterial strains BRG100 and 189. Fractionation of the culture supernatants of these strains showed that several fractions were significantly inhibitory against green foxtail seeds; this herbicidal activity was not observed in the fractions of nutrient control medium. A new peptidic siderophore, which we named **sideropseumin A**, was isolated from strain 189, but upon further assays no significant herbicidal activity was detected.

Herbicidal activity tests indicated that the most active components produced by strains BRG100 and 189 are water soluble peptides. A chromatographic method which allowed the separation of larger amounts of bioactive components produced by strains BRG100 and 189 was established. Namely, fractionation of the culture supernatants of these strains on a Diaion HP20 column showed that some fractions of the bacterial culture supernatants were significantly inhibitory against green foxtail seeds (herbicidal activity), and this activity was not observed in the fractions of nutrient medium. These fractions were produced in large volumes and further fractionated for isolation of herbicidal metabolites. However, further separations and herbicidal bioassays of fractions indicated a large dilution of activity; no single component displaying herbicidal activity was established.

In conclusion, our overall results suggest that both bacterial strains BRG100 and 189 could be useful agents to control soil borne plant pathogens due to production of a complex "blend" of antifungal metabolites, whose structures are derived from diverse amino acids. The herbicidal components produced by these bacterial strains appear to be a complex mixture of water soluble products with a broad range of molecular weights, extremely difficult to separate from the components of the nutrient medium. Solid medium was not a useful method to obtain potential herbicidal components. These results suggest that these bacteria have multiple mechanisms of action and that their herbicidal activity is due to multiple components secreted into the medium over a short growth period. In our hands ultrafiltration appeared to be the best method to separate herbicidal mixtures and may yield useful bioproducts upon lyofilization. However, until these products are further evaluated in appropriately formulated delivery systems their practical application remains unknown.

c) TECHNICAL REPORT

1. Objectives

Objectives of research project:

1) - to isolate and elucidate the chemical structures of antifungal and herbicidal natural products produced by two bacterial strains which are putative biocontrol agents (*Pseudomonas fluorescens*, strains 189 and BRG100 isolated by Dr. S. M. Boyetchko, at AAFC);

2) - to apply this knowledge to optimize through fermentation the production of bioactive natural products by those two bacteria.

2. Project activities, achievements, papers, and presentations

To protect intellectual property no oral presentations have been given.

Refereed publications:

Quail, J. W.; Ismail, N.; Pedras, M. S. C.; Boyetchko, S. M. 2002. Pseudophomins A and B, a class of cyclic lipodepsipeptides isolated from a *Pseudomonas* species. Acta Crystallographica, Section C: Crystal Structure Communications 58, 268-271

Pedras, M. S. C.; Ismail, N.; Quail, W.; Boyetchko, S. Structure, Chemistry, and Biological Activity of Pseudophomins A and B, a class of cyclic lipodepsipeptides isolated from the biocontrol bacterium *Pseudomonas fluorescens*. Submitted in July, 2002.

Pedras, M. S. C.; Ismail, N.; Boyetchko, S. Structure, Chemistry, and Biological Activity of Sideropseumin A a new peptidic siderophore from the biocontrol bacterium *Pseudomonas fluorescens*. In preparation.

3. Rationale

Microorganisms such as soil bacteria may be used for biological control and development of biorational products to reduce weed competition or deter plant diseases in crops. Antibiosis, that is production of antibiotic compounds or metabolites, appears to be a frequent mechanism utilized by biocontrol agents. These metabolites can inhibit microbial and weed growth and therefore control plant pathogens and weeds. Clear correlations have been established between antibiotic production and disease suppression. Diverse antibiotics have been implicated in the control of plant diseases and/or weeds by bacterial and fungal agents (Faull & Powell, 1995; Dowling & O`Gara, 1994); however, there is no clear correlation between the biocontrol species and the particular herbicidal or antifungal metabolite(s) produced. This implies that a detailed chemical analysis employing modern

spectroscopic techniques must be carried out in order to establish the chemical structures of the bioactive metabolites of any putative biocontrol agent. This aspect is addressed by our first objective.

The diversity of bioactive metabolites produced by biocontrol agents is highly dependent on environmental factors. In the laboratory, satisfactory production of metabolites requires that the producing strain be grown in synthetic media containing adequate nutrients. In particular, as demonstrated by a vast list of publications, the carbon sources may have a dramatic influence on the type and levels of antibiotics produced (Rosales et al., 1994). Thus, it is essential to have a good understanding of both growth requirements and antibiotic production before biocontrol agents can be employed. This aspect is addressed by our second objective.

4. Materials and Methods

4.1 General

Analytical TLC: (Merck, Kieselgel 60 F_{254}, aluminum sheets) 5×2 cm ×0.2 mm; mobile phase was dichloromethane - methanol (90:10) and compounds were visualized under UV light and by dipping the plates in a 5% aqueous (w/v) phosphomolybdic acid solution containing 1% (w/v) ceric sulfate and 4% (v/v) H_2SO_4, followed by heating.

HPLC analysis: performed with a liquid chromatograph equipped automatic injector, and diode array detector (wavelength range 190 - 600 nm), degasser, and a Hypersil ODS column (5 µm particle size silica, 4.6 i.d. × 200 mm), equipped with an in-line filter. Mobile phase, system A: 0.1% TFA in H_2O - 0.1% TFA in CH_3CN (40:60) to 100% CH_3CN containing 0.1% of TFA, for 30 min, linear gradient, 1.0 mL/min; system B: 0.1% TFA in H_2O - 0.1% TFA in CH_3CN (20:80) to 100% CH_3CN containing 0.1% of TFA, for 25 min, linear gradient, 1.0 mL/min.

Semi-preparative HPLC. performed with a liquid chromatograph synchronized with fraction collector and equipped with quaternary pump, automatic injector, degasser, and an EconoSphere® ODS semi-preparative column (10 µm particle size silica, 10.0 i.d. × 250 mm), equipped with an in-line filter with an isocratic elution (0.1 % TFA in H_2O–CH_3CN, 35:65) for 25 min, flow rate 5.0 mL/min.

Chiral GC analysis: GC analysis was carried out with a gas chromatograph, equipped with a *Chiral - Val* capillary column (25 m × 0.32 mm i.d., film thickness 0.2 µm, Alltech Chromatography), with helium as a carrier gas (flow 1.03 mL, measured at 70 °C); detector at 240 °C; injector at 220 °C; oven temperature 70 – 200 °C at 4 °C min^{-1}; samples were injected in split mode (ca. 50:1) with CH_2Cl_2 as solvent.

Specific rotations: $[\alpha]_D$ were determined on a DigiPol DP781 polarimeter, at room temperature, using a 1 mL, 10 cm cell at the indicated concentration (*c*; g/100 mL).

NMR analysis: NMR spectra were recorded on a Bruker AMX 300 or Bruker Avance 500 spectrometer; for ^1H (300 or 500 MHz), δ values were referenced to CD_3OD (CD_2HOD at 3.31 ppm), CD_2Cl_2 ($CDHCl_2$ at 5.32 ppm), $CDCl_3$ ($CHCl_3$ at 7.26 ppm), D_2O (HDO at 4.80 ppm), or $(CD_3)_2CO$ ($(CHD_2)_2CO$ at 2.05 ppm) and for ^{13}C (75.5 or 125.8 MHz) referenced to CD_3OD (49.15 ppm), CD_2Cl_2 (54.00 ppm), $CDCl_3$ (77.23 ppm) or $(CD_3)_2CO$ (206.68, 29.92). The multiplicities of ^{13}C NMR signals were determined utilizing J modulation experiments, HMQC and HMBC data.

HRMS analysis: mass spectra were obtained on a VG 70 SE mass spectrometer [high resolution (HR), electron impact (EI) or fast atom bombardment (FAB)], employing a solids and/or continuous flow probes.

4.2 Bacterial cultures: extractions and fractionation of extracts

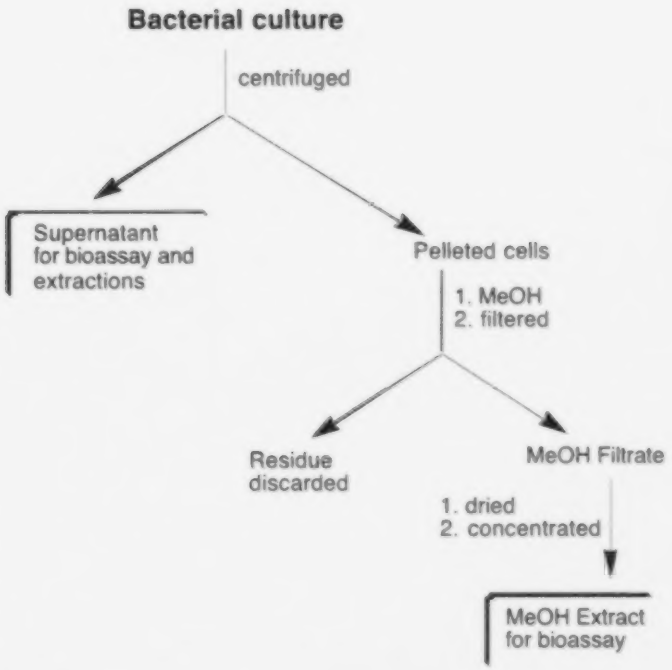
Cultures of *Pseudomonas fluorescens* BRG100 were streaked onto PAF plates and incubated for three to four days at room temperature (20-30 °C). Large scale bacterial cultures (twelve flasks containing 500 mL) were obtained by inoculating each flask with 200 µL of bacterial inoculum (20 mL nutrient broth sample inoculated with bacterial cells grown in PAF plates and incubated in a shaker at 150 rpm, 15 °C for 48 h). Control flasks containing only nutrient broth were prepared similarly. Nutrient medium was prepared according to Table 1 and incubated with bacterial inoculum at 15 °C, in a shaker at 150 rpm for 48 hours. Similar procedure was followed for culturing *P. fluorescens* strain 189.

Table 1. Composition of nutrient broth utilized for culturing strains BRG100 and 189

Constituents	Concentration (g / L)
Sodium phosphate	6.0
Ammonium chloride	1.0
Potassium dihydrogen orthophosphate	3.0
Sodium chloride	2.0
Magnesium sulfate	1.23
Thiamine hydrochloride	0.005
Calcium chloride	0.015
Glucose (carbon source) Trehalose (carbon source)	2.0 2.0

Bacterial cultures (batches of 5 L) were combined in a 10 L container and pumped into a flow through CEPA industrial centrifuge (40,000 rpm) at 250-300 mL/min. The pellet and supernatant were separated, the pellet was collected in methanol and the supernatant frozen immediately (Scheme 1). The pelleted cells were extracted with methanol, filtered through Whatman paper, the filtrate dried over sodium sulfate, and concentrated to dryness in a rotary evaporator.

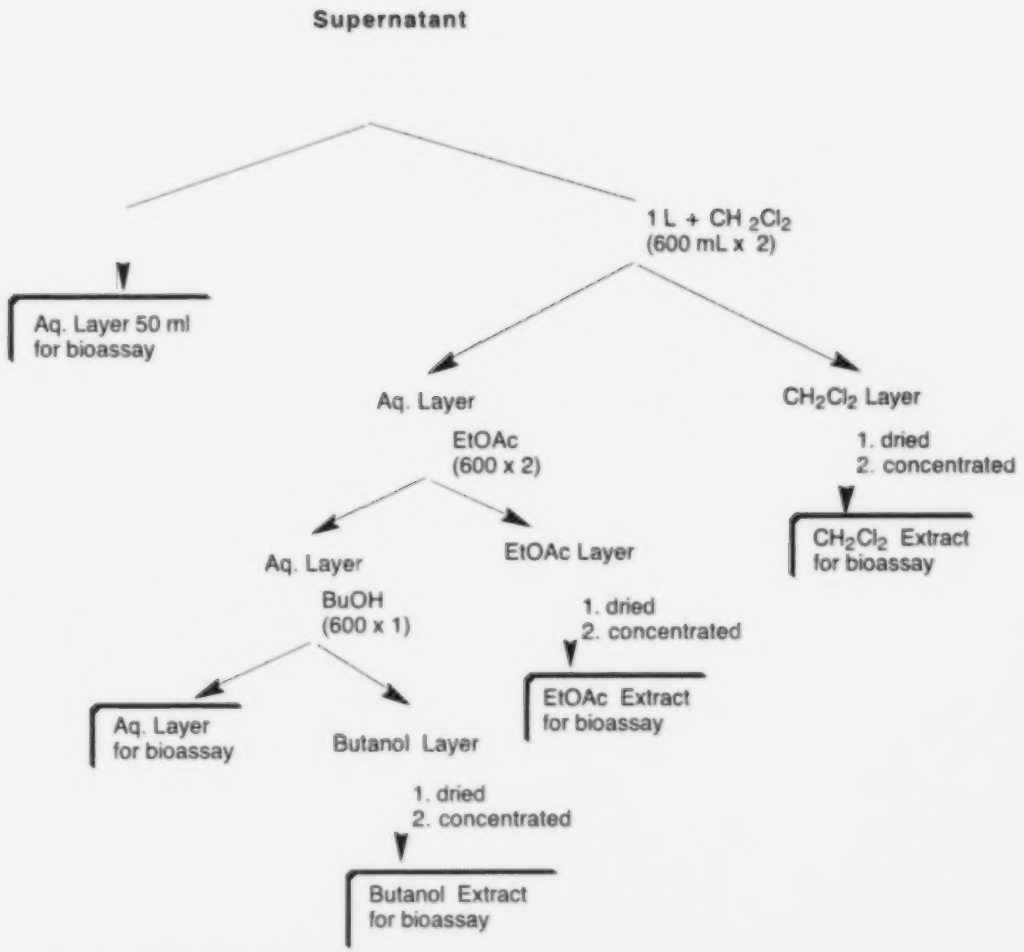
At the start of this project (March-Dec. 1999) we established whether the biological activity of the bacterial cultures was affected by processing culture supernatants through freeze-drying. Subsequently, having established that the biological activity was not affected, all culture supernatants were concentrated through freeze-drying to make the extraction process more efficient. Three types of culture broth containing as carbon sources either glucose, trehalose or sucrose were employed to culture strains BRG100 and 189.



Scheme 1. Flow chart for obtaining culture supernatants and cell extracts

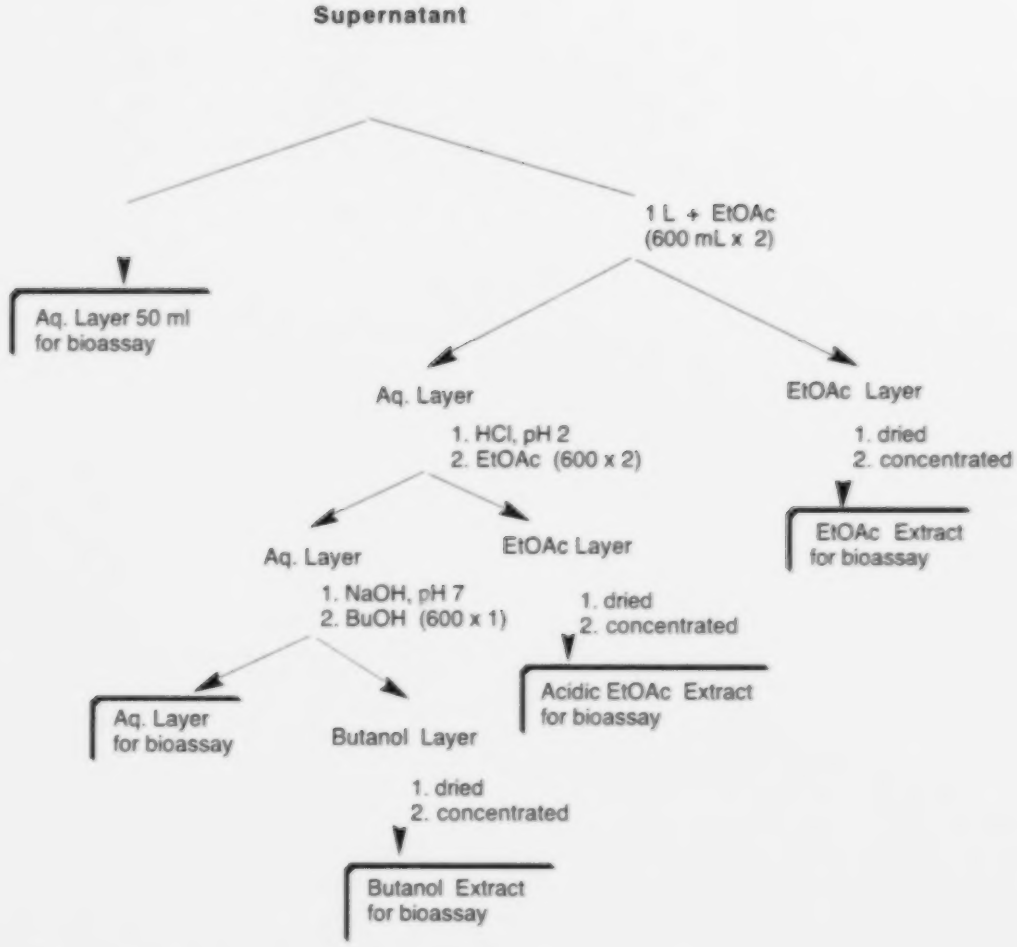
Preliminary bioassays with both strains suggested that the culture broth of strain BRG100 inoculated in medium containing either glucose or trehalose had stronger herbicidal activity than other combinations. Therefore, we concentrated our work on strain BRG100. Initially, cultures of BRG100 in glucose medium or trehalose medium were extracted with organic solvents separately according to protocols A and B shown in Schemes 2 and 3. Before extraction, samples of supernatant and nutrient broth (50 ml each) were stored separately for bioassays.

According to protocol A shown in Scheme 2, extractions of each supernatant were carried out first with dichloromethane, the organic phase was separated from the aqueous phase, dried over sodium sulfate, and concentrated in a rotary evaporator. The aqueous phase was then extracted with ethyl acetate, the ethyl acetate layer was separated and concentrated to dryness, while the aqueous phase was extracted with 1-butanol. The butanol phase was separated, dried over sodium sulfate, and concentrated to dryness. At the end of each extraction, the aqueous layer (50 ml) was stored for bioassay after complete evaporation of solvent and reconstitution with 50 ml of distilled water. Control broths were treated as the supernatants. The amounts of extracts obtained from each culture are shown in Table 2. Because the amounts of dichloromethane and ethyl acetate extracts utilizing this protocol were very small, and TLC and HPLC profiles of both extracts were similar, it was decided to combine these extracts and to modify this extraction protocol to obtain neutral and acidic extracts only with ethyl acetate, as shown in Scheme 3.



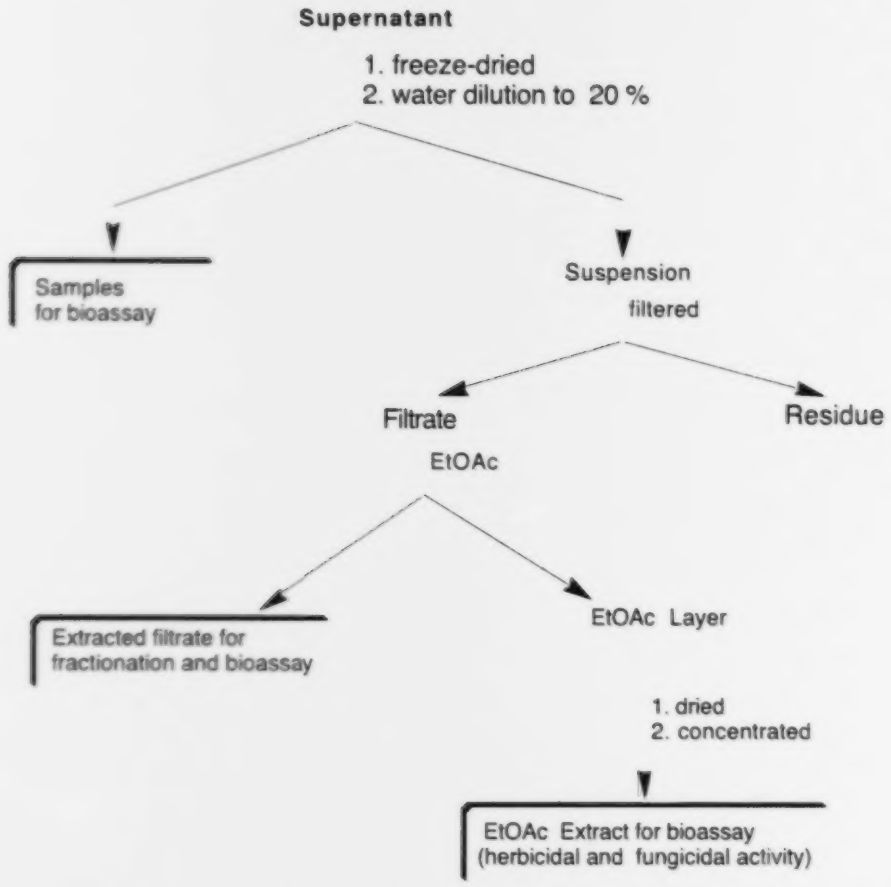
Scheme 2. Protocol A: flow chart for obtaining putative bioactive extracts of bacterial cultures.

According to protocol B shown in Scheme 3, extractions of each supernatant were carried out with ethyl acetate, the organic phase was separated from the aqueous phase, dried over sodium sulfate, and concentrated in a rotary evaporator. The aqueous phase was then acidified with HCl to pH 2 and extracted with ethyl acetate, the ethyl acetate layer was separated and concentrated to dryness, while the aqueous phase was neutralized with NaOH, and extracted with 1-butanol. The butanol phase was separated, dried over sodium sulfate, and concentrated to dryness. At the end of each extraction, the aqueous layer (50 ml) was stored for bioassay, after complete evaporation of water and reconstitution with 50 ml of distilled water. Control broths were extracted and treated similar to supernatants. Table 2 shows the amounts of cultures and extracts obtained during this project.



Scheme 3. Protocol B: flow chart for obtaining putative bioactive extracts of bacterial cultures.

The extracts of supernatants and controls obtained as shown in Schemes 2 and 3, as well as fractions and subfractions were analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) and bioassayed for antifungal and herbicidal activity. These results indicated that it would be advantageous to process strains BRG100 and 189 differently; subsequently, supernatants of strain BRG100 were processed according to protocol C and those of strain 189 were directly used for fractionation (Scheme 6).



Scheme 4. Protocol C: flow chart for obtaining extracts of freeze-dried culture supernatants of strain BRG100

4.2.1 Strain BRG100: fractionation, purification and chemical structure determination of new metabolites

4.2.1.1 Metabolites from extracts of supernatants

Ethyl acetate extracts (1.4 g) were obtained from culture supernatants (71 L of culture in glucose containing medium) followed by Protocol C. These extracts were combined and divided into five portions. Each portion was fractionated through column chromatography (silica gel Merck, grade 60, mesh size 230–400, 60 Å) using gradient elution with dichloromethane-methanol (CH_2Cl_2 to CH_2Cl_2–CH_3OH, 60:40), and the final fraction eluted with CH_3OH, 100 mL each). Fifty five fractions (five columns, 11 fractions per column) were collected, concentrated to dryness under vacuum, and

analyzed by TLC and HPLC. Those fractions determined to be similar by TLC and HPLC were combined and were bioassayed for antifungal activity, employing the mycelial radial growth assay. The fractions that showed antifungal activity were combined (124 mg) and were subjected to further fractionation on C_{18} reversed-phase silica gel (J. T. Baker, 40 μm) using a gradient elution with water–methanol (15:85). Fifteen sub-fractions were collected, similar fractions were combined and were bioassayed for antifungal and herbicidal activity.

HPLC analysis of the combined antifungal fractions showed two major peaks, with retention times of 16.7 min and 21.7 min, along with some minor peaks. This fraction was separated again on C_{18} reversed-phase silica gel (J. T. Baker, 40 μm) using a gradient elution of water-methanol (15:85) to yield pseudophomin B (40 mg), HPLC r_t = 21.7 min, as an white amorphous powder. Pseudophomin B was crystallized from aqueous acetonitrile to yield needle-like crystals. The remaining portion (54 mg) was further purified by reversed-phase semi-preparative HPLC (dissolved in water–methanol, 10:90 at a concentration 10 mg/mL) using mobile phase 0.1 % trifluoroacetic acid in acetonitrile–water, (65:35) for 35 min to yield pseudophomin A (35 mg), HPLC r_t = 16.7 min. Pseudophomin A was crystallized twice from dichloromethane–methanol (10:90). The purity of A and B was established by HPLC (0.1 % trifluoroacetic acid in water–acetonitrile, 40:60 to acetonitrile) for 30 min, linear gradient, and a flow rate = 1.0 mL/min).

Chemical characterization of Pseudophomin A

Pseudophomin A (**1**): HPLC r_t = 16.7 min (0.1 % trifluoroacetic acid in water–acetonitrile, 40:60 to acetonitrile for 30 min, linear gradient, and a flow rate = 1.0 mL/min).

$[\alpha]_D^{26}$ -22.6 (*c* 0.25, EtOH).

MS-FAB *m/z*: 1141 $(M+H)^+$, 1164 $(M+Na)^+$.

UV (MeOH) end absorption; IR (KBr) 3326, 2958, 1662, 1535 cm^{-1}.

^1H and ^{13}C NMR (see Table 4).

HRMS-FAB 1140.7139 (calcd for $C_{55}H_{98}N_9O_{16}$, 1140.7132), 857.4997 (calcd for $C_{39}H_{69}N_8O_{13}$, 857.4984), 728.4561 (calcd for $C_{34}H_{62}N_7O_{10}$, 728.4558), 609.3857 (calcd for $C_{31}H_{53}N_4O_8$, 609.3863), 496.3038 (calcd for $C_{25}H_{42}N_3O_7$, 496.3022) 413.2647 (calcd for $C_{21}H_{37}N_2O_6$, 413.2651), 284.2230 (calcd for $C_{16}H_{30}NO_3$, 284.2225).

Chemical characterization of Pseudophomin B

Pseudophomin B (**2**): HPLC r_t = 21.7 min (0.1 % trifluoroacetic acid in water–acetonitrile, 40:60 to acetonitrile for 30 min, linear gradient, and a flow rate = 1.0 mL/min).

$[\alpha]_D^{26}$ -14.7° (*c* 0.40, EtOH).

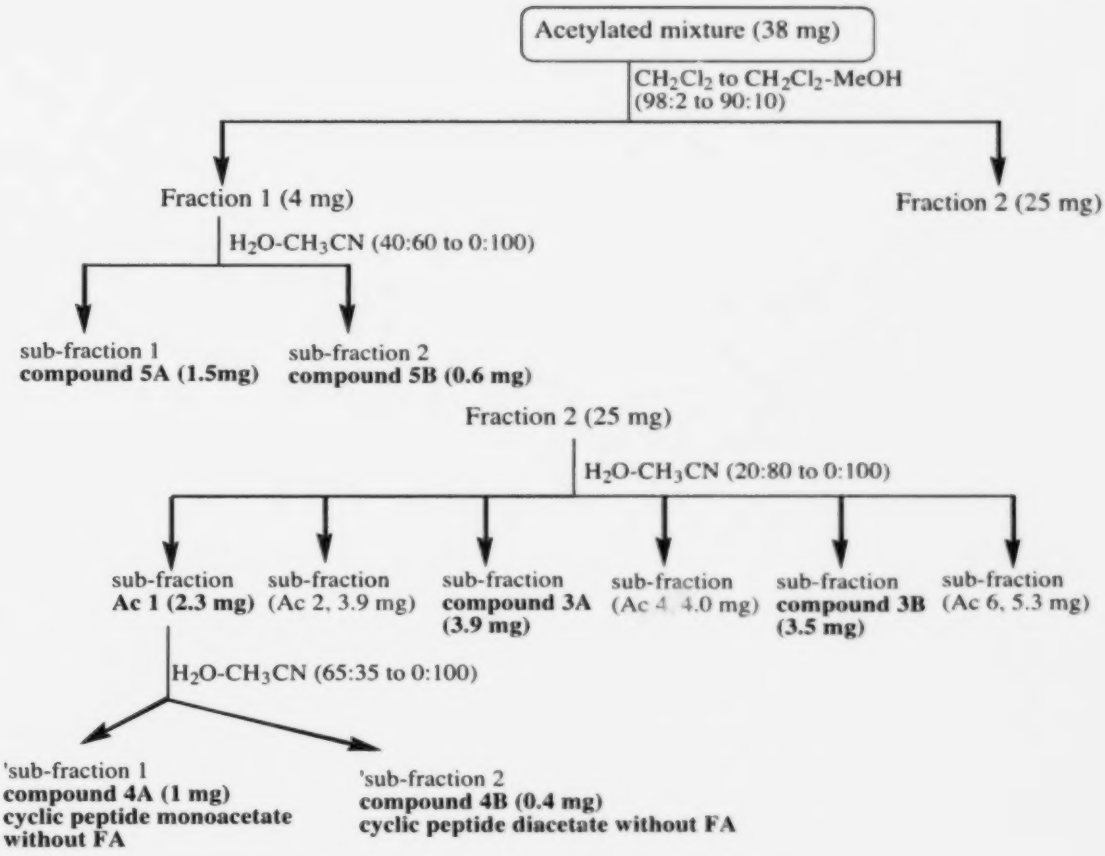
UV (MeOH) end absorption; IR (KBr) 3325, 2958, 1661, 1533 cm^{-1}.

^1H and ^{13}C NMR (see Table 3).

HRMS-FAB *m/z*: 1168.7456 (calcd for $C_{57}H_{102}N_9O_{16}$, 1168.7444), 857.4980 (calcd for $C_{39}H_{69}N_8O_{13}$, 857.4984), 728.4562 (calcd for $C_{34}H_{62}N_7O_{10}$, 728.4558), 637.4183 (calcd for $C_{33}H_{57}N_4O_8$, 637.4176),

524.3330 (calcd for $C_{27}H_{46}N_3O_7$, 524.3335), 441.2970 (calcd for $C_{23}H_{41}N_2O_6$, 441.2964), 312.2531 (calcd for $C_{18}H_{34}NO_3$, 312.2538).

Acetylation


Acetylated mixture (38 mg)
CH_2Cl_2 to CH_2Cl_2-MeOH (98:2 to 90:10)
Fraction 1 (4 mg)
Fraction 2 (25 mg)
H_2O-CH_3CN (40:60 to 0:100)
sub-fraction 1
compound 5A (1.5mg)
sub-fraction 2
compound 5B (0.6 mg)
Fraction 2 (25 mg)
H_2O-CH_3CN (20:80 to 0:100)
sub-fraction Ac 1 (2.3 mg)
sub-fraction (Ac 2, 3.9 mg)
sub-fraction compound 3A (3.9 mg)
sub-fraction (Ac 4, 4.0 mg)
sub-fraction compound 3B (3.5 mg)
sub-fraction (Ac 6, 5.3 mg)
H_2O-CH_3CN (65:35 to 0:100)
'sub-fraction 1
compound 4A (1 mg)
cyclic peptide monoacetate without FA
'sub-fraction 2
compound 4B (0.4 mg)
cyclic peptide diacetate without FA

Scheme 5. Flow chart for further fractionation of acetylated mixture.

A portion of an antifungal fraction containing pseudophomins A and B (38 mg) was dissolved in acetic anhydride (1.3 mL) and pyridine (2.6 mL) and kept overnight at room temperature. The reaction mixture was diluted with toluene and concentrated under reduced pressure. Separation of the reaction mixture by CC (silica gel Merck, grade 60°A) using gradient elution with dichloromethane–methanol, (CH_2Cl_2, to CH_2Cl_2–CH_3OH, 98:2, 20 mL each, and final fraction with CH_2Cl_2–CH_3OH, 90:10) afforded thirteen fractions, which were analyzed by TLC (dichloromethane–methanol, 98:02) and HPLC (0.1 % TFA in water–acetonitrile, 40:60, to acetonitrile for 35 min, linear gradient, and a flow rate = 1.0 mL/min). The HPLC chromatogram of fraction 1 (4 mg) showed two peaks, retention time 10.4 min and 15.3 min (Scheme 5). The fraction was further purified by reversed-phase semi-preparative HPLC, (dissolved in acetonitrile, at a concentration 20 mg/mL) using mobile phase 0.1 %

trifluoroacetic acid in water-acetonitrile, (40:60 to 0:100) linear gradient, and a flow rate = 5.0 mL/min) for 20 min to yield compound **5A** (1.5 mg) and compound **5B** (0.6 mg) as shown in Scheme 5.

The HPLC chromatogram of fraction 2 (25 mg) of the acetylated mixture showed three major peaks with retention times 2.2, 13.8 and 18.4 min (Scheme 5). This fraction was further purified by reversed-phase semi-preparative HPLC (dissolved in acetonitrile, at a concentration 150 mg/mL) using mobile phase 0.1 % trifluoroacetic acid in water-acetonitrile, (20:80 to 0:100) linear gradient, and a flow rate of 5.0 mL/min for 25 min, to yield six sub-fractions (Ac 1, Ac 2, Ac 3, Ac 4, Ac 5, and Ac 6, concentrated to dryness under vacuum and analyzed by TLC and HPLC). The sub-fraction Ac 3 (r_t = 13.8 min, compound **3A**, 3.9 mg) and sub-fraction Ac 5 (r_t = 18.4 min, compound **3B**, 3.5 mg) contained pure compounds and spectroscopic data were collected.

The HPLC chromatogram of sub-fraction Ac1 (2.3 mg) showed two major peaks, retention time 10.1 min and 13.2 min, along with some minor peaks using (0.1 % TFA in water–acetonitrile, 65:35, to acetonitrile for 25 min, linear gradient, and a flow rate = 1.0 mL/min). This sub-fraction was further purified by reversed-phase semi-preparative HPLC (dissolved in acetonitrile-methanol, 50:50, v/v at a concentration 23 mg/mL) using mobile phase 0.1 % trifluoroacetic acid in water-acetonitrile (65:35 to 0:100) for 25 min, linear gradient, and a flow rate = 5.0 mL/min) to yield compound **4A** (1 mg, r_t 10.1 min) and compound **4B** (0.4 mg, r_t 13.2). The purity of **4A** and **4B** was established by HPLC (0.1 % trifluoroacetic acid in water-acetonitrile, 65:35 to acetonitrile) for 25 min, linear gradient, and a flow rate = 1.0 mL/min). Identical derivatives were identified through HPLC analysis when pure compounds; pseudophomins A and B were acetylated separately.

Chemical characterization of compound 5A

^{1}H NMR ($CDCl_3$) δ: 0.88, 0.94 (d, 6H, J = 5.9 Hz), 1.24 (br s), 1.54 (m), 1.63 (m, 2H), 1.69 (m), 2.07 (s, 3H), 2.52 (d, 2H, J = 6.1 Hz), 3.74 (s, 3H), 4.62 (m, 1H), 5.14 (dq, 1H, J = 12.2, 5.9 Hz).
^{13}C NMR ($CDCl_3$) δ: 14.4, 21.5, 22.3, 23.0, 23.1, 25.2, 25.6, 29.6, 30.1, 32.1, 34.4, 41.8, 42.0, 51.1, 52.6, 71.8, 170.0, 171.1, 173.8.

MS-FAB m/z: 358 $(M+H)^+$.

HRMS-FAB m/z: 358.2587 (calcd for $C_{19}H_{36}NO_5$ 358.2593).

Chemical characterization of compound 5B

^{1}H-NMR ($CDCl_3$) δ: 0.87, 0.94 (d, 6H, J = 5.9 Hz), 1.24 (br s), 1.58 (br s), 2.03 (s, 3H), 2.50 (d, 2H, J = 6.1 Hz), 3.72 (s, 3H), 4.62 (m, 1H), 5.14 (m, 1H), 6.07 (d, 1H, J = 8.1 Hz).
MS-FAB m/z: 386 $(M+H)^+$.

HRMS-FAB m/z: 326.2679 (calcd for $C_{19}H_{36}NO_3$ (M-Ac-H_2O)+ 326.2695).

Chemical characterization of compound 3A

MS-FAB m/z: 1249 (M+H)+.

HRMS-FAB m/z: 1248.7320 (calcd for $C_{61}H_{102}N_9O_{18}$ $(M+H)^+$ 1248.7342).

Chemical characterization of compound 3B

^{13}CNMR($CDCl_3$) δ : 10.7, 12.3, 14.5, 16.1, 16.6, 18.8, 19.0, 21.2, 21.3, 21.6, 21.7, 22.2, 23.0, 23.3, 23.5, 23.5, 23.7, 24.7, 24.9, 25.0, 25.2, 25.7, 25.7, 29.6, 29.8, 30.0, 32.5, 33.8, 35.6, 35.7, 36.3, 38.8, 39.9, 41.3, 44.0, 52.6, 52.7, 54.3, 54.5, 54.9, 57.1, 62.3, 62.6, 63.2, 64.2, 64.5, 68.9, 73.2, 168.9, 169.8, 170.3, 171.0, 171.4, 171.6, 171.6, 172.9, 173.2, 173.4, 174.1, 174.6, 174.8.

MS-FAB *m/z*: 1277 $(M+H)^+$.

HRMS-FAB *m/z*: 1276.7704 (calcd for $C_{63}H_{106}N_9O_{18}$ $(M+H)^+$ 1276.7655).

Chemical characterization of compound 4A

^{1}H-NMR (acetone d_6) δ: 0.84-0.94 (m), 1.01 (3H, *J* = 6.3 Hz), 1.36 (3H, *J* = 5.9 Hz), 2.14 (s, $CO\underline{CH_3}$), 5.15 (m, 1H), 5.45 (dq, 1H, *J* = 10.3, 5.8 Hz), 6.84 (d, 1H, *J* = 10 Hz), 7.17 (d, 1H, *J* = 8.2 Hz), 7.28 (d, 1H, *J* = 6.3 Hz), 7.94 (d, 1H, *J* = 8.2 Hz), 8.03 (m, 1H), 8.48 (m, 2H), 9.42 (m, 1H).

C NMR (acetone d6) 10.3, 11.7, 15.6, 15.7, 18.1, 20.9, 21.7, 22.5, 22.5, 23.1, 24.6, 24.7, 24.8, 25.8, 25.9, 32.5, 34.8, 36.5, 40.4, 41.2, 53.9, 54.6, 55.9, 56.4, 56.4, 57.8, 61.5, 62.6, 62.7, 63.7, 69.8, 169.4, 169.8, 170.1, 171.1, 171.9, 172.2, 172.8, 176.5, 173.5, 173.5.

MS-FAB m/z: 881 (M+Ac)+, 839 (M+H)+.

HRMS-FAB m/z: 881.4988 (calcd for C41H69N8O13 (M+Ac)+ 881.4984), 839.4883 (calcd for C39H67N8O12 (M+H)+ , 839.4878).

Chemical characterization of compound 4B

1H-NMR (acetone d6) 0.86 (m 0.88 (m 0.92 (d) 0.93 (d, J = 6.5 Hz), 0.98 (d, J = 6.5 Hz), 1.00 (d, J = 6 Hz),1.25, 1.36 (m), 1.42 (d, 3H, J = 6 Hz), 1.51 (m), 1.69 (t, 1H), 1.86 (m), 1.91, 1.98 (m), 2.00, 2.03 (s, COCH3), 2.05 (s, COCH3), 3.98 (t, J = 7.5 Hz), 4.31 (m), 4.41 (d, 2H, J = 6 Hz), 4.44 (m, 1H), 4.66 (m, 1H), 4.88 (m, 1H), 5.39 (m, 1H), 7.12 (d, 1H, J = 7.5 Hz), 7.56 (d, 3H, J = 8.8 Hz), 7.74 (d, 1H, J = 4 Hz), 8.03 (d, 1H, J = 5.5 Hz), 8.12 (br s, 1H), 8.82 (br s, 1H).

^{13}C NMR (acetone d_6) δ: 10.8, 11.4, 15.6, 15.3, 15.5, 18.2, 20.3, 20.3, 21.0, 21.9, 22.5, 23.0, 24.7, 24.8, 25.0, 25.8, 25.9, 35.9, 36.6, 40.5, 40.8, 52.6, 53.2, 53.4, 53.9, 57.2, 57.7, 61.2, 61.2, 63.6, 63.9, 70.0, 169.6, 169.6, 169.6, 169.8, 170.3, 170.5, 172.7, 172.7, 173.3, 174.9, 178.6, one CH_2 signal under solvent peak.

MS-FAB *m/z*: 923 $(M+2Ac)^+$, 881 $(M+Ac)^+$, 839 $(M+H)^+$.

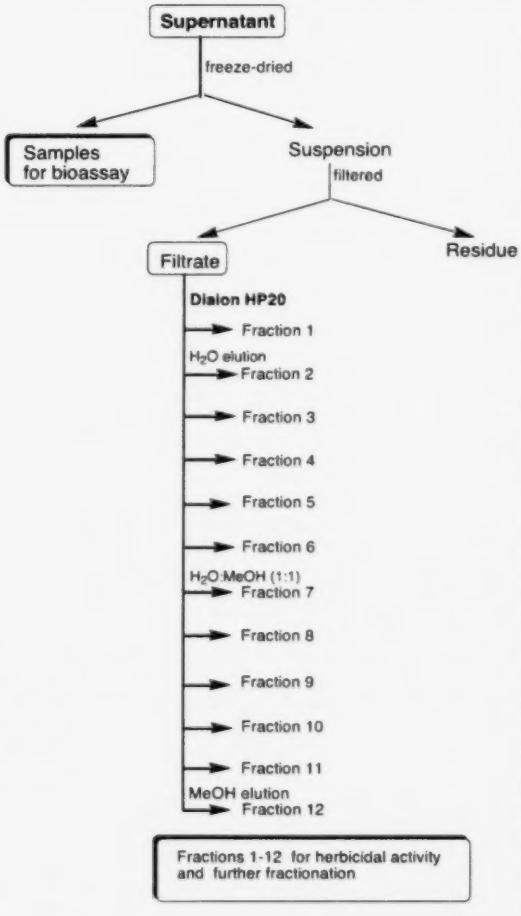
HRMS-FAB *m/z*: 923.5111 (calcd for $C_{43}H_{71}N_8O_{14}$ $(M+2Ac)^+$ 923.5089), 881.4996 (calcd for $C_{41}H_{69}N_8O_{13}$ $(M+Ac)^+$ 881.4984), 839.4883 (calcd for $C_{39}H_{67}N_8O_{12}$ $(M+H)^+$, 839.4878).

Hydrolysis and derivatization

Degassed 6 M HCl (0.5 mL) was added to compound 5A (0.6 mg, 0.0016 mmol), the tube was quickly sealed and was partially immersed in a silicon oil-bath at 110-115 °C, for 24 hr. The hydrolyzed product was concentrated to dryness in a rotary evaporator. Similar procedure was adopted for compound 5B.

Derivatization steps were performed in a Reacti® vial with close fitting screw caps. A solution of hydrolyzed product in acetyl chloride–2-propanol (10:90 v/v, 200 µL) was heated to 110 °C under argon for 60 min. The mixture of isopropyl ester derivative was cooled and concentrated in vacuum. Dichloromethane (200 µL) and trifluoroacetic anhydride (TFAA, 100 µL) were added to the isopropyl ester derivative while cooling the mixture in ice. The reaction was carried out at room temperature for 15 hr. The N,O-trifluoroacetyl isopropyl ester derivative was concentrated with a slow stream of argon and excess reagent was removed. The N,O-trifluoroacetyl isopropyl esters of compound 5A was analyzed by GC followed by GC-MS. Standards of pure amino acids (D-leucine and DL-leucine, Sigma Aldrich Chemical Co.) were prepared similarly.

4.2.1.2 Fractionation of supernatants with herbicidal activity



Previous bioassays established that extracted non-freeze-dried and freeze-dried supernatants had similar herbicidal activity. Subsequently, to facilitate isolation of metabolites, fractionation of culture supernatants in large volumes was carried out on freeze-dried culture supernatants. Thus, the aqueous layers (extracted supernatants) collected after extraction with ethyl acetate as shown in Scheme 4, were concentrated to remove residual solvent and were further fractionated as described below. The following paragraphs summarize the lengthy experimental procedure required for the fractionation of culture supernatants to obtain constituents with herbicidal activity.

Culture supernatants (60 L) of strain BRG100 (glucose medium) collected after extraction with ethyl acetate were freeze-dried and the concentrated syrup was divided into 30 portions. Each portion (equivalent to 2 L of non-freeze-dried culture supernatants) was fractionated on a Diaion HP20 column. The culture supernatant was adsorbed onto the Diaion HP20 resin overnight, a glass column was loaded with the resin (4.5 i.d. × 50 cm) and the aqueous phase collected to yield fraction 1; fractions were further eluted with gradient elution water–methanol (distilled water 1000 mL to water–methanol, 50:50, 1000 mL, and final fraction with methanol, 1000 mL). Two hundred and seventy six fractions (23 columns, 12 fractions per column) were collected (200 mL each), freeze-dried

Scheme 6. Flow chart for obtaining fractions of freeze-dried culture supernatants of strain BRG100 and strain 189

and /or concentrated to dryness under reduced pressure (Scheme 6).

The fractions of culture supernatants and controls were analyzed by high performance liquid chromatography (HPLC) with the following mobile phase: 0.1 % TFA in water to water–acetonitrile, 50:50 to 0:100 acetonitrile, for 38 min, linear gradient and a flow rate of 1.0 mL/min.

The aqueous culture fractions were tested for herbicidal activity against green foxtail. The total amount of active culture fraction 7 (900 mg from 23 columns) was divided into even portions; one portion (200 mg) was subjected to further fractionation by FCC over Sephadex LH 20 (Fluka) using a gradient elution methanol-water (80:20 to 0:100), 50 mL each. Nine sub-fractions were collected, concentrated to dryness under vacuum, bioassayed for herbicidal activity (green foxtail and analyzed by HPLC (0.1 % TFA in water to water–acetonitrile, 50:50 to 0:100 acetonitrile, for 38 min, linear gradient and a flow rate of 1.0 mL/min).

4.2.1.3 Solid media bacterial cultures: extraction and chemical analysis of extracts

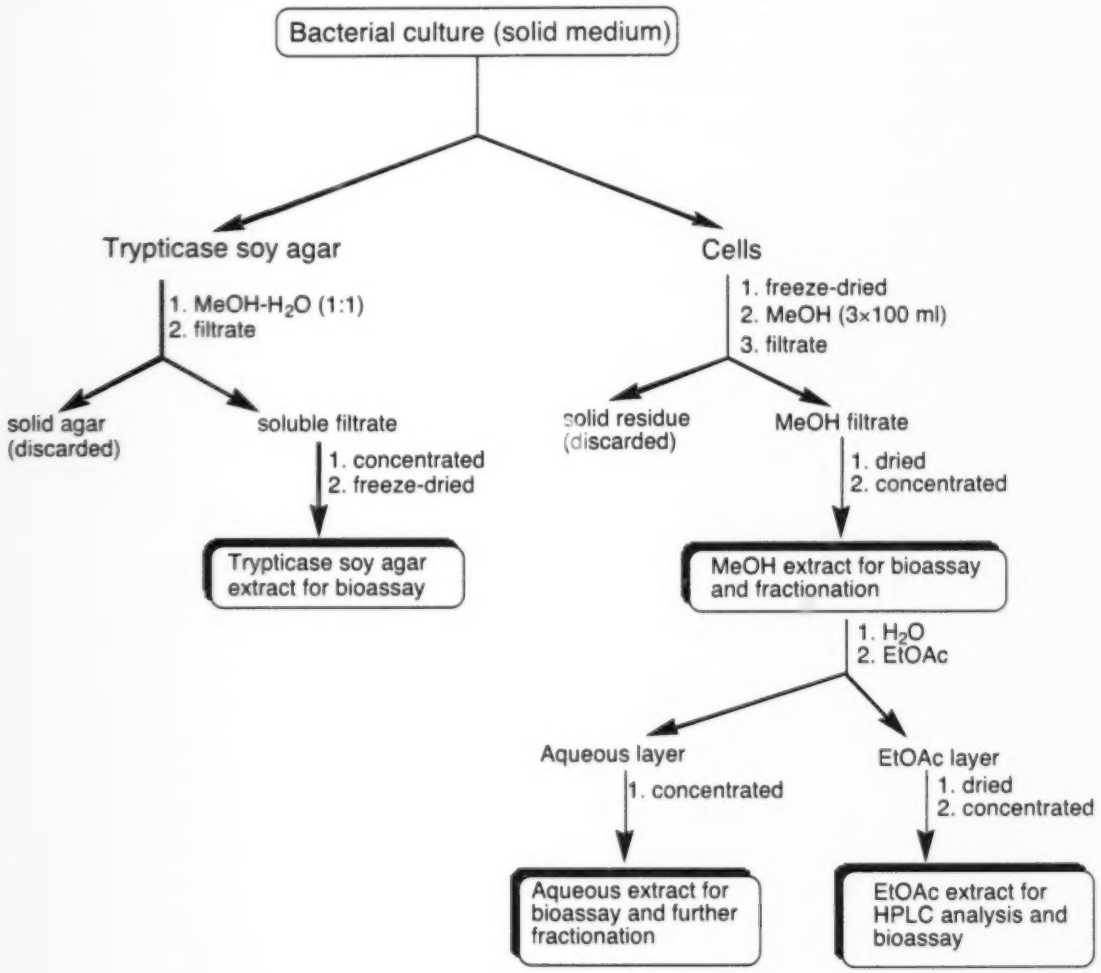
Cultures of *P. fluorescens* strain BRG100 were grown on two different solid media; trypticase soy agar (TSA) medium (trypticase soy broth 30 g, Becton Dickinson with agar 15 g, Difco) in 1 L of distilled water and autoclaved for 15 min, hereon referred to as "100% TSA medium" and "50% TSA medium" (trypticase soy broth 15 g, with agar 15 g) in 1 L of distilled water and autoclaved for 15 min.

Fresh colonies of *P. fluorescens* strain BRG100 were grown in a PAF plate and incubated for three to four days at room temperature (20-30 °C). The bacterial lawn was scraped off from the surface with a poly plate spreader and suspended in a Falcon® tube containing sterile water (10 mL), homogenized and 100 µL dispensed per plate containing solid TSA, as described above. The bacterial suspensions were spread evenly and incubated for 48 h, 72 h and 96 h at room temperature (20-30 °C). Total 48 plates (2 media × 3 incubation time × 8 plates each).

After incubation, the bacterial cells were scraped off agar plates and suspended in distilled water (50 mL) and subsequently freeze-dried. The dried cells were extracted with methanol (3×100 mL), filtered through Whatman filter paper, the filtrate dried over sodium sulfate, and concentrated to dryness in a rotary evaporator. A portion of methanol extract was saved for herbicidal activity against green foxtail. The methanol extract was diluted with water and then extracted with ethyl acetate, the ethyl acetate layer was separated and concentrated to dryness, and the aqueous layer was evaporated to dryness. Control plates contain 100% and/or 50% TSA (without bacterial inoculation) were incubated similarly (Scheme 7).

The solid trypticase soy agar was extracted with water – methanol (50:50 v/v, 1 L), and filtered through Whatman filter paper. The filtrates were initially concentrated on a rotary evaporator to remove residual methanol and finally freeze-dried. The extracts obtained as shown in Scheme 7, as well as corresponding controls were analyzed by HPLC. Methanol extracts and the aqueous extracts were analyzed with the following mobile phase: 0.1 % TFA in water to water–acetonitrile, 50:50 to 0:100 acetonitrile, for 38 min, linear gradient and a flow rate of 1.0 mL/min. The HPLC

chromatograms of ethyl acetate extracts were obtained with the following mobile phase: 0.1 % TFA in water-acetonitrile, 40:60 to 0:100 acetonitrile, for 30 min, linear gradient, and a flow rate of 1.0 mL/min. All the extracts obtained were bioassayed against green foxtail.



Scheme 7. Flow chart for obtaining extracts from bacterial cultures strain BRG100 grown on solid trypticase soy agar medium (TSA).

4.2.1.4 Ultrafiltration of culture supernatants

A portion of cell free culture supernatants (50 mL) was filtered through a membrane with a molecular weight cut off (MWCO) 10,000, mounted in a Jumbosep centrifugal concentrator (membrane surface area 15.2 cm^2, Pall Gelman Sciences), centrifuged at 3720 rpm at 25 °C for 10 min, or until ca. 1/2 of

the volume was filtered. The first upper portion was further filtered through a membrane MWCO 30,000 in a macrosep centrifuge device, at 6,500 rpm for 10 min while the first lower portion was filtered through a membrane MWCO 3,000 in a macrosep filtration device, at 3720 rpm for 45 min. A portion of the first upper portion was diluted to original volume with water and the ultrafiltration was repeated to yield second upper and lower fractions. Additional ultrafiltration through a membrane MWCO 1,000 was conducted. To be used as controls for bioassays, culture medium was filtered through membranes with MWCO 30,000 - 1,000 Da similarly.

4.2.2 Strain 189: fractionation, purification and chemical structure determination of new metabolites

4.2.2.1 Metabolites from extracts of supernatants

Ethyl acetate extracts (0.3 g) were obtained from culture supernatants (34 L of culture in glucose containing medium) following Protocols A and B. The HPLC chromatogram showed one major compound and the UV spectrum resembles to the UV spectra of pseudophomins A and B. Due to lower activity and production in the culture, this extract was not used further.

4.2.2.2 Fractionation of supernatants with herbicidal activity and isolation of metabolites

Bacterial cultures (40 L) of strain 189 grown in glucose medium were freeze-dried to reduce the volume (Scheme 6). The suspension formed during the process was filtered off and the precipitate was removed. The freeze-dried filtered cultures were divided into 20 portions. Each portion (equivalent to 2 L of non-freeze-dried culture supernatants) was adsorbed on Diaion HP20 resin overnight, a glass column loaded with the resin (4.5 i.d. × 50 cm) and the culture collected to yield fraction 1; fractions were further eluted with gradient elution water–methanol (distilled water 1000 mL to water–methanol, 50:50, 1000 mL, and final fraction with methanol, 1000 mL). Two hundred and forty fractions (20 columns, 12 fractions per column) were collected (200 mL each), each fraction of first column was combined with the fractions of other nineteen columns. Finally, the combined twelve fractions (4000 mL each fraction) were freeze-dried and /or concentrated to dryness under reduced pressure (Scheme 6). The residues obtained from culture supernatants and from controls were analyzed by HPLC, as described above. The residues were tested for herbicidal activity against green foxtail.

The residues of culture fractions with biological activity and obtained in sufficient amounts were selected for further fractionation. The HPLC chromatogram of active culture fraction **7** showed one major peak along with other minor peaks. The total amount of culture fraction 7 (600 mg from 20 columns) was divided into three portions. Each portion was fractionated through column chromatography on C_{18} reversed-phase silica gel using a gradient elution of water–methanol (90:10), final fractions were eluted with CH_3OH, (50 mL each). Thirty three sub-fractions (three columns, 11 fractions per column) were collected, concentrated to dryness, and analyzed by HPLC. The fractions determined to be similar were combined to yield three major sub-fractions. The HPLC chromatogram of the sub-fraction 1 (350 mg) showed few UV active peaks, while sub-fraction 2 (200 mg) showed one medium strong peak along with minor ones. The sub-fraction 3 (68 mg) showed one major

compound (also present in sub-fraction 2) with other minor peaks. This sub-fraction was re-chromatographed on C_{18} reversed-phase silica gel using a gradient elution of water–methanol (95:05 to 92:08 to 90:10), final fraction eluted with CH_3OH, (50 mL each). Sixty three sub-fractions (three columns, 21 fractions per column) were collected, concentrated to dryness under vacuum, and analyzed by HPLC. Compound A (24 mg) was obtained as brown powder; HPLC r_t = 10.7 min, (water to water–acetonitrile, 50:50 to 0:100 acetonitrile, for 38 min, linear gradient and a flow rate = 1.0 mL/min). The HPLC chromatogram of culture fraction **12** showed one major peak along with minor peaks. The fraction 12 (140 mg from 20 columns) was divided into two portions and each portion was fractionated through column chromatography on C_{18} reversed-phase silica gel using a gradient elution of water-methanol (90:10). Twenty four sub-fractions (two columns, 12 fractions per column) were collected and analyzed by TLC and HPLC. The HPLC chromatogram of compound B (7.6 mg) showed single peak with a retention time 12.3 min, mobile phase: 0.1 % TFA in water to water–acetonitrile, 50:50 to 0:100 acetonitrile, for 38 min, linear gradient and a flow rate = 1.0 mL/min. The structure of compound B was established by NMR and mass spectroscopy and confirmed by synthesis as reported in the literature (Nakano, J. 1971) and described below.

Chemical characterization of compound A

HPLC r_t = 10.7 min, (water to water–acetonitrile, 50:50 to 0:100 acetonitrile, for 38 min, linear gradient and a flow rate = 1.0 mL/min).

UV (H_2O) λ_{max}: 404, 322 (sh), 265 (sh), 231 nm.

^{1}H NMR (D_2O-H_2O, 1:9; pH 4) δ: 0.95 (d, 3H, *J* = 7 Hz), 1.07 (d, 3H, *J* = 6 Hz), 1.11 (d, 3H, *J* = 6 Hz), 1.37 (d, 3H, *J* = 7 Hz), 1.76 (br m), 2.07 (br m), 2.07 (m), 2.23 (m), 2.54 (d, 2H, *J* = 13.5 Hz), 2.70 (d, 2H, *J* = 11.5 Hz), 2.75 (m), 2.81 (m), 2.90 (m), 2.97 (br s), 3.26 (br s), 3.46 (dt, 1H, *J* = 7, 13 Hz), 3.61 (m), 3.73 (d, 1H, *J* = 7 Hz), 3.99 (br s), 4.07 (d, *J* = 7.5 Hz), 4.43 (d, 1 H *J* = 9.5 Hz), 4.50 (dt, 1H, *J* = 7, 13 Hz), 4.57 (d, 1H, *J* = 11 Hz), 5.52 (br s, 1H), 6.09 (s), 6.72 (s), 6.84 (d, *J* = 7 Hz), 6.90 (br s), 7.21 (d, *J* = 10 Hz), 7.75 (d, *J* = 8 Hz), 7.80 (s), 7.89 (s), 8.04 (s), 9.08 (s), 11.43 (s).

^{13}C NMR (D_2O) δ: 15.4 (q), 15.6 (q), 15.7 (q), 16.0 (q), 20.0 (t), 21.9 (t), 23.4 (t), 26.9 (t), 28.4 (t), 28.7 (t), 30.7 (t), 31.6 (t), 35.4 (t), 37.9 (t), 39.6 (t), 39.8 (t), 47.4 (d), 50.7 (d), 51.3 (d), 51.3 (t), 52.6 (d), 52.9 (d), 53.1 (t), 53.4 (d), 54.5 (d), 54.9 (d), 56.2 (d), 68.3 (d), 75.3 (d), 95.1 (d), 108.1 (d), 113.3 (s), 114.1 (s), 132.1 (s), 139.0 (d), 148.6 (s), 152.4 (d), 153.2 (s), 153.8 (d), 162.8 (s), 167.2 (s), 169.2 (s), 170.7 (s), 171.6 (s), 171.7 (s), 171.8 (s), 173.2 (s), 175.4 (s), 175.4 (s), 176.2 (s), 177.2 (s), 177.2 (s), 177.4 (s).

MS-FAB *m/z*: 1271 $(M+H)^+$

HRMS- FAB *m/z*: 1270.5920 (calcd for $C_{53}H_{80}N_{19}O_{18}$, 1270.5928).

Chemical characterization of compound B

HPLC r_t = 12.3 min, (0.1 % TFA in water to water–acetonitrile, 50:50 to 0:100 acetonitrile, for 38 min, linear gradient and a flow rate = 1.0 mL/min).

^{1}H NMR (CD_3OD) δ: 2.07 (s, 3H), 2.41 (s, 3H), 2.51 (br s), 3.51 (t, 2H, *J* = 6.5 Hz), 7.83 (s, 1H), 7.88 (s, 1H).

^{13}C NMR (CD_3OD) δ: 17.7 (q), 24.0 (q), 33.8 (t), 39.7 (t), 59.4 (t), 109.0 (s), 134.6 (s), 155.8 (d), 163.1 (s), 164.2 (d), 167.5 (s).

MS-EI *m/z*: 280

HRMS-EI *m/z*: 280.0995 (calcd for $C_{12}H_{16}N_4O_2S$, 280.0993).

Synthesis of compound B

Compound B was synthesized according to Scheme 9. An aqueous solution (7.6 mL) of thiamine hydrochloride (1 g) containing NaOH (0.36 g) was stirred in ice bath. After 30 min, diethylamine (1 mL) followed by an aqueous solution (3 mL) of I_2 (1.6 g) and KI (0.95 g) was added gradually to the reaction mixture. After complete addition, the reaction mixture was kept at room temperature for 75 min. Excess amine was removed from the reaction mixture on a rotary evaporator under reduced pressure and the reaction mixture extracted with chloroform (10 mL × 3). The $CHCl_3$ layer was washed with small amount of water, dried over anhydrous Na_2SO_4, evaporated to dryness on a rotary evaporator to yield an oily residue (125 mg). FCC of this residue afforded fourteen fractions. The first two fractions were eluted with chloroform, and further eluted with chloroform-methanol gradient. The fractions were concentrated on a rotary evaporator and analyzed by thin layer chromatography (TLC) ($CHCl_3$–MeOH, 90:10). The structures of S-amino thiamine **3** (9 mg), pyrimido[4,5-c][1,2,6]-10H-thiadiazocine **4** (20 mg), and sulfinic acid ester **5** (54 mg) were assigned by NMR and mass spectroscopy.

Chemical characterization of S-amino thiamine (3)

^{1}H NMR ($CDCl_3$) δ: 1.14 (t, 6H, J = 7 Hz), 2.01 (s, 3H), 2.49 (s, 3H), 3.07 (m, 6H) 3.64 (brs, 1H), 3.74 (brs, 1H), 4.39 (d, 1H, J = 15 Hz), 4.60 (d, 1H, J = 15 Hz), 6.06 (brs, 2H), 7.95 (s, 2H).

MS-EI *m/z*: 297; MS-CI *m/z*: 370

HRMS-FAB *m/z*: 370.1919 (calcd for $C_{16}H_{28}N_5O_3S$, $(M+1)^+$, 370.1912).

Chemical characterization of pyrimido[4,5-c][1,2,6]-10H-thiadiazocine (4)

^{1}H NMR ($CDCl_3$) δ: 1.96 (s, 3H), 2.49 (s, 3H), 2.81 (t, 2H, J = 6 Hz), 4.18 (t, 2H, J = 6.5 Hz), 4.39 (s, 2H), 5.97 (brs, 1H), 7.88 (s, 1H), 8.05 (s, 1H).

^{13}C NMR ($CDCl_3$) δ: 19.8 (q), 25.7 (q), 33.7 (t), 39.8 (t), 76.3 (t), 109.1 (s), 116.3 (s), 146.5 (s), 156.0 (d), 161.9 (s), 163.8 (d), 168.2 (s).

^{1}H NMR (CD_3OD) δ: 2.04 (s, 3H), 2.39 (s, 3H), 2.83 (t, 2H, J = 6.5 Hz), 4.12 (t, 2H, J = 6.5 Hz), 4.44 (s, 2H), 7.87 (s, 1H), 8.05 (s, 1H).

MS-EI *m/z*: 280.

HRMS-EI 280.0993 (calcd for $C_{12}H_{16}N_4O_2S$, 280.0993), 159.0364 (calcd for $C_6H_9NO_2S$, 159.0354), 123.0833 (calcd for $C_6H_9N_3$, 123.0796).

Chemical characterization of sulfinic acid ester (5)

^{1}H NMR ($CDCl_3$) δ: 2.02 (s, 3H), 2.47 (s, 3H), 2.70 (m, 1H), 2.92 (dd, 1H, J = 16, 6.5 Hz), 4.15 (d, 1H, J = 15 Hz), 4.71 (td, 1H, J = 9 Hz), 4.92 (m, 1H), 5.01 (d, 1H, J = 15 Hz), 5.96 (brs, 1H), 7.87 (s, 1H), 8.27 (s, 1H).

MS-EI *m/z*: 296.

HRMS-EI 296.0940 (calcd for $C_{12}H_{16}N_4O_3S$, 296.0943), 123.0752 (calcd for $C_6H_9N_3$, 123.0796).

Synthesis of 5,6-Dihydro-2,7-dimethyl-formyl-8-(2-hydroxyethl)-pyrimido [4,5-c][1,2,6]-10H-thiadiazocine (6)

Pyrimido[4,5-c][1,2,6]-10H-thiadiazocine **4**, (9.7 mg) was dissolved in H_2O by heating and the resulting solution was kept at 4 °C. After three days the solution was extracted with chloroform (2 mL × 3). The aqueous layer was evaporated to dryness on a rotary evaporator and yielded compound **6**; 5,6-Dihydro-2,7-dimethyl-formyl-8-(2-hydroxyethl)-pyrimido[4,5-c][1,2,6]-10H-thiadiazocine was analyzed by ^{1}H NMR.

^{1}H NMR (CD_3OD) δ: 2.07 (s, 3H), 2.41 (s, 3H), 2.51 (br s), 3.52 (t, 2H, J = 6.5 Hz), 7.83 (s, 1H), 7.88 (s, 1H).

4.2.2.3 Ultrafiltration of culture supernatants of strain 189

A portion of cell free culture supernatants (50 mL)of strain 189 was ultrafiltered through a membrane of molecular weight cut off 10K in a jumbosep centrifugal concentrator as reported for strain BRG100. The upper and lower portions were tested for herbicidal activity against green foxtail.

4.3 Herbicidal activity bioassays

For bioassay-guided isolation only protocols A, B and C were used. Protocols B and C require smaller amounts of sample; protocol D was used for determination of herbicidal activity of extracts and purified compounds.

Protocol A: Bioassay plates were prepared with agar medium (0.9%) containing supernatant broth (30% v/v), culture supernatant, upper and lower portions of ultrafiltered culture supernatant (30% v/v), or corresponding control medium (30% v/v). Seeds of green foxtail were sterilized (Javex - Tween - water, 10: 0.5 : 89.5, for 10 min) placed on agar plates, and plates were sealed and placed in an incubator at 20 °C. Germination and/or root growth was measured after 7 days; controls were used to determine percentage of germination inhibition. Seeds that did not germinate were not considered in seedling length measurements. Six replicate plates containing eight seeds per plate were employed in each bioassay.

Protocol B: Seeds of green foxtail were sterilized (Javex-Tween 20-H_2O, 33:0.5:66.5, for 10 min) soaked in a solution of extract (2 mg/mL) or compound (0.5 mg/mL) prepared in sterile H_2O or H_2O–CH_3CH_2OH (90:10) for 30 min, and the coated seeds were placed in Petri plates lined with sterile moistened Whatman filter paper. The plates were sealed and placed in an incubator at 20 °C under darkness. Germination and/or root length was measured after 7 days. Controls (seeds treated with solvent) were used to determine the percentage of inhibition. Seeds that did not germinate were not considered in seedling length measurements. Three replicate plates containing five seeds per plate were employed in each bioassay.

Protocol C: Seeds of green foxtail were sterilized as above, germinated for three days on moist filter paper, and the germinated seeds were soaked in a solution of extract (2 mg/mL) or compound (0.5 mg/mL) prepared in sterile H_2O or H_2O–CH_3OH (90:10) for 15 min, and the coated seeds were placed

in Petrie plates lined with sterile moistened Whatman filter paper. The plates were sealed and placed in an incubator at 20 °C under darkness. Growth and/or root length was measured after 7 days. Controls (seeds treated with solvent) were used to determine the percentage of inhibition. Three replicate plates containing five seeds per plate were employed in each bioassay.

Protocol D: Seeds of green foxtail were sterilized as above; 24-well multidish plates prepared containing agar medium (0.9%, 1.5 mL per well) amended with EtOAc extract or pseudophomins A or B dissolved in DMSO. The assay was conducted in five replicates, one seed per well was placed on agar plates, and plates were sealed and placed in an incubator at 20 °C in darkness. Germination and/or root length was measured after 7 days. Controls (non-amended medium) were used to determine the percentage of inhibition.

4.4 Antifungal activity bioassays

Bioassays to determine antifungal activity were carried out with the following plant pathogens: *Phoma lingam* (Tode ex Fr.) Desm., sexual stage = *Leptosphaeria maculans* (Desm.) Ces. et de Not.; *Alternaria brassicae*, (Berk.) Sacc., isolate AB 11-1; *Sclerotinia sclerotiorum* (Lib.) de Bary, clone #33; *Rhizoctonia solani* Kuhn, isolate AG 2-1. *Phoma lingam* virulent isolate BJ 125 was grown on V8 agar under continuous light; after 15 days the fungal spores were collected and stored at -20 °C as previously reported (Pedras and Khan, 1996). *Alternaria brassicae*, isolate AB 11-1 was grown on potato dextrose agar (PDA) at 25 °C under continuous light for 13 days (Pedras et al., 1998). *Sclerotinia sclerotiorum* and *Rhizoctonia solani*, isolate AG 2-1 were grown on potato dextrose agar (PDA), incubated under continuous light at 25^0 C for 3 and 4 days respectively (Pedras and Khan, 2000).

Protocol A: The antifungal activity of cell free bacterial cultures was tested by spore germination bioassay in 6–well multidish plates (potato dextrose medium plus supernatant, 1:1, v/v, 2 mL per well). A plug of mycelia cut from the edge of active culture, was placed upside down at the centre of each well (the plates were incubated under continuous light at 25 °C, bioassays were conducted in triplicate). The radial growth of mycelial plug was measured after 6 days of incubation. Control plates containing potato dextrose broth plus bacterial medium (1:1, v/v) were prepared and incubated in similar way. The percentage of inhibition was calculated relative to control plates.

Protocol B: The extracts of cell-free bacterial cultures obtained according to protocol A and protocol B were tested against the same four fungal species by radial growth assay. Dichloromethane, ethyl acetate, and acidic ethyl acetate extracts (2 mg/mL) were dissolved in dichloromethane - methanol (1 : 1, v/v) and butanol extracts were dissolved in dichloromethane - methanol - water (4.4:4.6:1.0 v/v/v). while fractions, sub-fractions of ethyl acetate extracts or pseudophomins A and B were tested at a concentration of 0.5 mg/mL; dissolved in CH_2Cl_2–MeOH (9 : 1, v/v). Agar medium (prepared with V8 juice for *P. lingam* and *A. brassicae*, or potato dextrose for *S. sclerotiorum* and *R. solani*) was poured into glass Petrie plates (6 cm) and allowed to cool to room temperature. Solutions (2 mL/plate) were applied onto the agar plate to make a uniform film on the agar surface, and the solvent was allowed to evaporate under a laminar flow hood. After four hours, the plates were inoculated with mycelial plugs of actively growing cultures of the pathogen. Control plates containing solvent only were prepared and incubated in similar way. The percentage of inhibition was calculated relative to control plates by measuring the diameter of mycelial growth.

5. Results and Discussion

The production of bioactive natural products by the bacterium *P. fluorescence* strains BRG100 and 189 was investigated utilizing a bioassay-guided isolation approach (Pedras, 1998).

5.1 Liquid medium bacterial cultures

Initially three types of culture broth containing as carbon sources either glucose, trehalose or sucrose were employed to culture BRG100 and 189. Preliminary bioassays with both strains suggested that the culture broth of strain BRG100 inoculated in glucose or trehalose medium had stronger herbicidal activity than other combinations. We followed both protocols A (Scheme 2) and B (Scheme 3) to extract the bacterial cultures. However, because the amounts of dichloromethane and ethyl acetate extracts utilizing protocols A were very small, and TLC and HPLC profiles of both extracts were similar. protocol B (Scheme 3) was followed to extract the culture supernatants. However, because freeze-dried culture supernatants showed bioactivity (herbicidal and antifungal) similar to that of non-freeze-dried supernatants (that is freeze-drying did not destroy bioactivity), freeze-drying was employed so that larger volumes of culture supernatants could be processed.

Table 2. Cultures and extracts of strains BRG100 and 189

Strain **Culture medium (volume)**	**Culture medium (volume)** (protocol A-C)	**CH_2Cl_2; EtOAc extract (mg)**	**Acidic EtOAc extract (mg)**	**Butanol extract (mg)**
BRG100	Glucose (22 + 71 L)	548; 1400	748	3378
BRG100	Trehalose (18 L)	171	48	490
BRG 100	Sucrose (2 L)	11	--------	375
189	Glucose (34 L)	324	--------	785
189	Trehalose (14 L) Trehalose (25 L)	187 not processed	114	855
189	Sucrose (14 L)	88	104	968
Control	Glucose (7 L)	29	17	725
Control	Trehalose (15 L)	27	12	472
Control	Sucrose (4 L)	9	--------	190

In addition, because supernatants extracted according to Scheme 2 showed herbicidal activity against green foxtail similar to that of non-extracted cultures, a simpler extraction protocol was devised as summarized in protocols C (Scheme 4). Subsequently, supernatants were extracted according to Scheme 4, and the remaining aqueous layer freeze-dried and fractionated as reported above (4.2.1.2).

The amounts of extracts obtained from each culture are shown in Table 2; these extracts were used to determine antifungal and herbicidal activity. Corresponding controls were extracted similarly.

Chemical analysis of each extract was carried out by TLC and HPLC. Representative chromatograms of different extracts of strains BRG100 and 189 are shown on Figures 1 and 2, respectively. None of the chromatograms of control broths and butanol extracts of bacterial cultures contained a significant number of UV active constituents (chromatograms not shown).

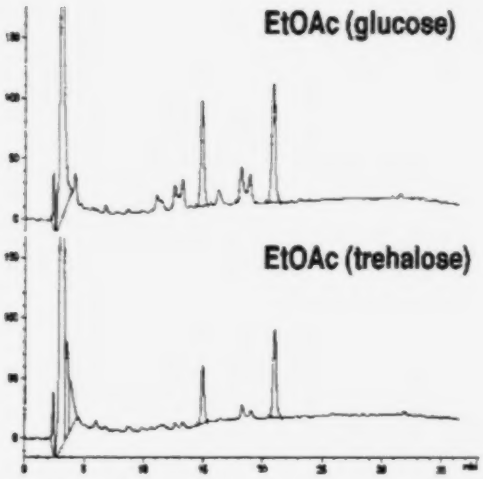


Figure 1. HPLC chromatograms (206 nm) of ethyl acetate extracts of cultures of strain BRG100 grown in glucose (Glu-EtOAc) and trehalose (Tre-EtOAc) media extracted according to protocol B.

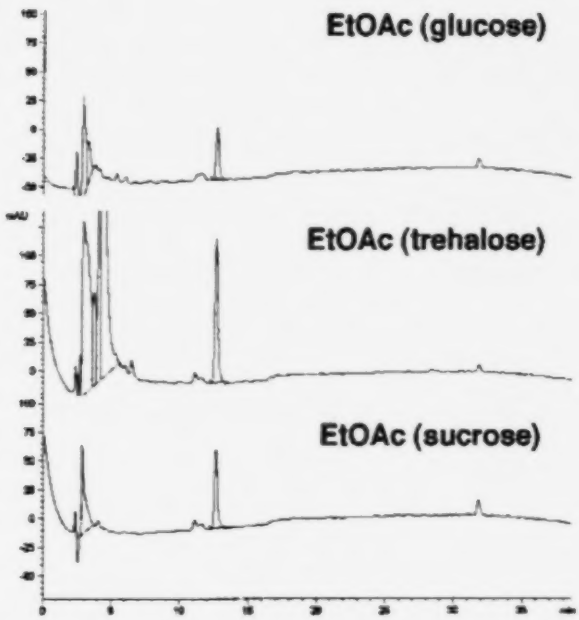


Figure 2. HPLC chromatograms (206 nm) of ethyl acetate extracts (EtOAc) of cultures of strain BRG100 grown in glucose, trehalose, and sucrose media extracted according to protocol B.

Comparison of the HPLC chromatograms of dichloromethane and ethyl acetate extracts of strains BRG100 and 189 with TLC chromatograms of same extracts indicated that only a small number of the extract constituents absorbed UV-Visible radiation (206-600 nm; peaks not present in control extracts). The chromatograms indicated that strain BRG100 produced a higher number of UV-active metabolites than strain 189. Moreover, comparison of the HPLC chromatograms of ethyl acetate extracts of strain BRG100 and 189 obtained from non-freeze-dried supernatant with those of freeze-dried supernatants indicated they were similar.

5.1.1 Strain BRG100: fractionation, purification and chemical structure determination of new metabolites

Scheme 4 was followed to extract the culture supernatants of strain BRG100, and the remaining aqueous layer was freeze-dried and further fractionated as described in 4.2.1.2. Ethyl acetate extracts of culture supernatants of strain BRG100 were fractionated through silica gel (described in 4.2.1); the fractions were assayed for antifungal activity, and analyzed by TLC, HPLC (Figure 3), and NMR spectroscopy. Comparison of HPLC chromatograms of antifungal fractions with those of ethyl acetate extracts and corresponding TLC chromatograms indicated that the major UV active components present in the extracts were also present in these fractions. After multiple attempts to separate these components utilizing a variety of solid and mobile phases, the best separation of this antifungal fraction was achieved utilizing C_{18} reversed-phase silica gel.

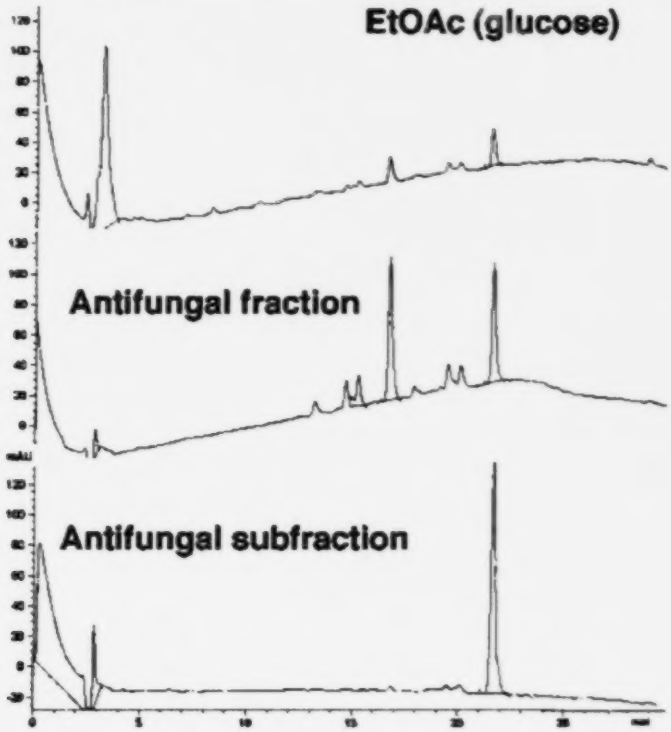


Figure 3. HPLC chromatograms of ethyl acetate (EtOAc) extract, antifungal fraction and antifungal subfraction of culture supernatants of strain BRG 100 grown in glucose medium

5.1.1.1 New metabolites from extracts of supernatants

EtOAc extracts from culture supernatants of *P. fluorescens* strain BRG100 were combined, concentrated, and fractionated by column chromatography; resulting fractions were combined and bioassayed for antifungal and herbicidal activity, employing fungal radial growth and root growth assays, as described in materials and methods. No substantial herbicidal activity was detected in any of the fractions. The fractions that showed antifungal activity were combined and were subjected to further fractionation on C_{18} reversed-phase silica gel. HPLC analysis of the combined antifungal fractions showed two major peaks, with retention times of 16.7 min and 21.7 min (system A, Figure 3), along with some minor peaks. This fraction was further separated on C_{18} reversed-phase silica gel to yield pseudophomin B (**2**, 40 mg, r_t = 21.7 min, white amorphous powder), which was crystallized from aqueous acetonitrile to yield needle-like crystals. The remaining portion (54 mg) was further purified by reversed-phase HPLC to yield pseudophomin A (**1**, 35 mg, r_t = 16.7 min, white amorphous powder, Figure 4), which was recrystallized from CH_2Cl_2–MeOH.

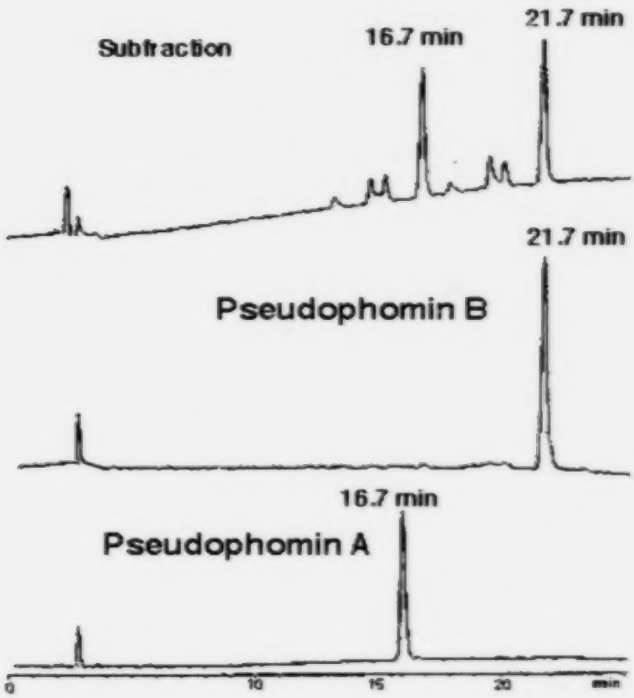
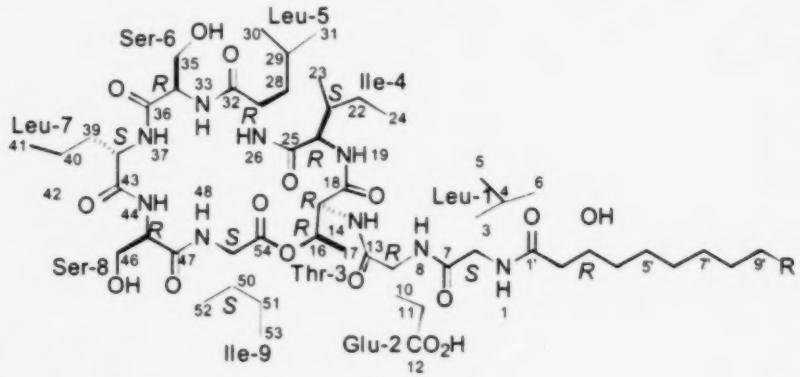


Figure 4. HPLC chromatograms of ethyl acetate (EtOAc) extract of culture supernatants of strain BRG100 grown in glucose, an antifungal subfraction, and pseudophomins A(16.7 min) and B (21.7 min) obtained from further separation of antifungal subfraction

Pseudophomin B was obtained as optically active ($[\alpha]_D$ –15, EtOH) colorless needles; both the ^1H and ^{13}C NMR spectroscopic data of pseudophomin B were indicative of a lipopeptidic structure. In the

positive-ion FAB–MS spectrum, its molecular ion peak appeared at *m/z* 1191 and 1169 indicating ions resulting from addition of sodium $[M+Na]^+$ and proton $[M+H]^+$, respectively. The positive ion HRMS–FAB showed a peak at *m/z* 1168.7456 suggesting a molecular formula of $C_{57}H_{102}N_9O_{16}$ (calcd *m/z* 1168.7444, $[M+H]^+$), consistent with both the ^1H and ^{13}C NMR spectra. In addition, the ^1H NMR spectrum showed nine signals at δ_H 7.10–9.44 likely due to NH protons. Detailed analysis of the HMQC, HMBC, and COSY spectra showed the presence of nine amino acids: three leucine (Leu), two isoleucine (Ile), two serine (Ser), one threonine (Thr) and one glutamic acid (Glu) residues. After subtraction of nine amino acid residues ($C_{45}H_{79}O_{14}$) from the molecular formula of pseudophomin B, the remaining fragment of the molecule contained 12 carbons, 23 hydrogens and two oxygen atoms. Furthermore, the NMR data showed that this fragment contained one aliphatic methyl (CH_3), one carbinol methine (CHOH), nine methylenes (CH_2) and a carbonyl (CO) carbon, indicative of a 12-carbon fatty acid chain. Complete analysis of the data suggested that pseudophomin B had structure **2**, which was confirmed by X-ray crystallographic analysis (Quail et al., 2002).

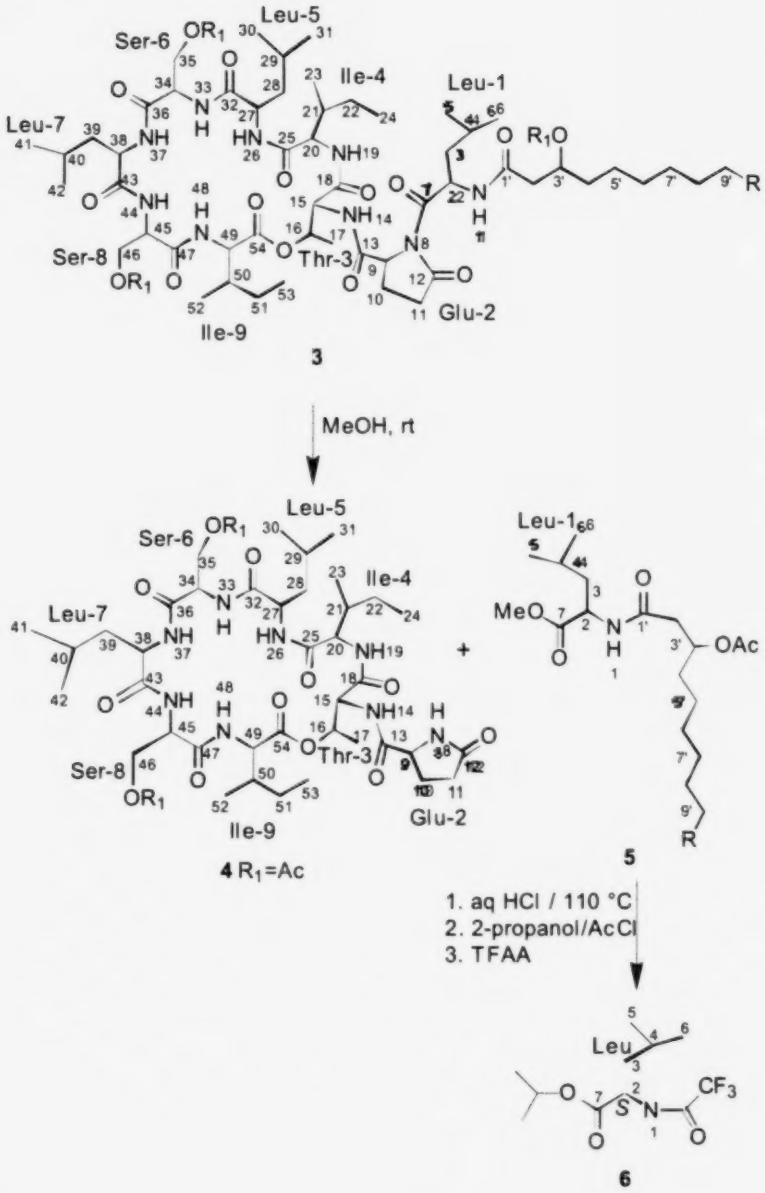
Pseudophomin A (**1**, 35 mg) was obtained as an optically active ($[\alpha]_D$ –23, EtOH) white powder. The positive-ion MS-FAB spectrum exhibited ions at *m/z* 1164 and 1141 indicating addition of sodium $[M+Na]^+$ and proton $[M+H]^+$ to the molecular ion, respectively. The positive ion HRMS–FAB showed a peak at *m/z* 1140.7139 indicating a molecular formula of $C_{55}H_{98}N_9O_{16}$ (calcd *m/z* 1140.7132), and suggesting that the compound only differed from pseudophomin B ($C_{57}H_{102}N_9O_{16}$) by two methylene units. Comparison of the NMR spectra (Tables 3 and 4) of pseudophomin A (**1**) with those of B (**2**) showed the same number of amino acid residues and MS-FAB data indicated that both compounds only differed in the β–hydroxy fatty acid residue.



1 R=CH_3
2 R=$CH_2CH_2CH_3$

*Figure 5. Chemical structures of pseudophomins A (**1**) and B (**2**)*

Although the relative configurations of the hydroxy acid and amino acid residues of pseudophomins A and B could be established by X-ray crystallography (Quail et al., 2002), the absolute stereochemistry required chemical degradation and derivatization as follows (Scheme 8).



Scheme 8. Determination of absolute configuration of pseudophomins A (1) and B (2)

Pseudophomin A (**1**) or B (**2**) was acetylated to triacetates **3a** (**3**, R = CH_3, R_1 = Ac) or **3b** (**3**, R = $CH_2CH_2CH_3$, R_1 = Ac), respectively, dissolved in MeOH, and separated by reversed-phase HPLC to yield **4** (R = Ac; HPLC r_t = 2.3 min) and **5a** (**5**, R = CH_3, HPLC r_t = 3.8 min) in the case of

pseudophomin A, or **4** and **5b** (**5**, R = $CH_2CH_2CH_3$, HPLC r_t = 5.3 min) in the case of pseudophomin B (Scheme 8). The chemical structure of depsipeptide **4B** was established by analysis of the NMR data (Table 5) and MS spectroscopic data. The γ-lactam moiety present in **4B** is likely to result from formation of the mixed anhydride of Glu-2 during acetylation, followed by nucleophilic displacement of acetate to form the C(12)-N(8) imide bond, which upon methanolysis yields compounds **4A, 4B** and **5**. Product **5** was hydrolyzed in aqueous HCl and derivatized to yield leucine derivative **6**. This unique selective degradation allowed the unambiguous determination of the absolute configuration of the amino acid residue Leu-1, due to selective cleavage of the N(8)-C(7) bond. Although there are many examples of γ-lactam formation in proteins and peptides (Bateman et al., 1990; Baldwin et al., 1990; Abraham and Podell, 1981), to the best of our knowledge this is the first time that selective imide bond cleavage is used to determine the absolute configuration of a cyclic depsipeptide.

A literature search showed that pseudophomin A (**1**) is a diastereomer of massetolide A and pseudophomin B (**2**) is a diastereomer of massetolide C (Gerard et al., 1997). The few crystal structures of cyclic lipodepsipeptides determined to date include WLIP, tensin, and amphisin (Sorensen et al., 2001). Interestingly, the chemical structure of pseudophomin A, including the absolute configuration of each stereogenic center, is similar to that of WLIP, except for the D-Ile-4 residue which in WLIP is replaced with D-Val residue (Han et al., 1992; Mortishire-Smith et al., 1991). WLIP is a lipodepsipeptide produced by P. reactans which inhibits browning of mushrooms caused by *P. tolaasii*. Mushroom caps treated with various concentrations of a crude WLIP sample, and later inoculated with bacterial concentrations higher than the threshold value, did not develop the symptoms of the disease (Soler-Rivas et al., 1999). To date several lipodepsipeptides displaying broad bioactivity spanning from phytotoxic to antiviral, antibacterial, and antifungal, have been isolated from diverse Pseudomonas species (Balkovec, 1994). Recently, it was demonstrated that the cytotoxic mode of action of some lipodepsipeptides is due to formation of ion channels that are freely permeable to divalent cations thus promoting passive transmembrane ion fluxes and causing cell necrosis (Hutchison and Gross, 1997). It is worthy to note that pseudophomin B is substantially more active against the phytopathogens *P. lingam* and *S. sclerotiorum* than pseudophomin A (Table 22), and is likely to be the main component responsible for the antifungal activity of EtOAc extracts of strain BRG100. These results suggest that potential changes on transmembrane ion fluxes are not likely to be the sole molecular mechanism involved in the somewhat selective fungal growth inhibition caused by pseudophomins.

5.1.1.2 Fractionation of supernatants with herbicidal activity

Since the herbicidal activity of the ethyl acetate extracted supernatants and non-extracted culture supernatants were comparable, it was concluded that the herbicidal constituents were not efficiently extracted with organic solvents. Several attempts were made to separate these water soluble components with aqueous solvents and compatible solid supports. Eventually we established a separation procedure using a Diaion HP20 column, as described in 4.2.1.2 and summarized in Scheme 6. The nutrient medium was fractionated similar to the culture supernatants. Table 6 shows the amount of residues of each fraction. It was found that the first and second fractions contained mainly salts and sugars used in the medium preparation. However, this method of fractionation and desalting of culture supernatants by Diaion HP20 was proven to be the best method. The culture fractions were tested for herbicidal activity (Tables 11-12). Further fractionation of the bioactive fractions of the Diaion HP20

column lead to fractions with greatly reduced herbicidal activity; for this reason further separation was not carried out.

Table 3. ^{1}H and ^{13}C NMR Assignments of Pseudophomin B (**2**) in CD_3OD

Residue	C #	$^{13}C^{a}$ δ (ppm) (mult)	^{1}H δ (ppm) multiplicity (*J* in Hz)	HMBC[c]	Residue	C #	$^{13}C^{a}$ δ (ppm) (mult)	^{1}H δ (ppm) multiplicity (*J* in Hz)	HMBC[c]
C_{12} HO-acid	1′	174.2 (s)				22	25.7 (t)	1.45, m 1.29[b]	
	2′	43.6 (t)	2.45 d (7)	3′, 1′		23	16.3 (q)	1.01, d (6.5)	
	3′	68.8 (d)	4.08, m	1′, 4′		24	9.9 (q)	0.97	
	4′	39.7 (t)	1.74, m			25	174.9 (s)		
	5′	25.7 (t)	1.29[b]		Leu-2	NH		7.28, d, (7)	
	6′	29.7 (t)	1.29[b]			27	62.5 (d)	3.76, dt (8, 3.5)	29, 32
	7′	29.7 (t)	1.29[b]			28	37.5 (t)	1.45, m	
	8′	32.1 (t)	1.29[b]			29	35.2 (d)	2.12, br m	
	9′	29.7 (t)	1.29[b]			30	21.7 (q)	0.91	
	10′	29.5 (t)	1.29[b]			31	22.7 (q)	0.92, d (6)	
	11′	22.7 (t)	1.29[b]			32	172.3 (s)		
	12′	13.5 (q)	0.92		Ser-1	NH		7.60, d (4)	
Leu-1	NH		7.75, d			34	56.4 (d)	4.39, m	32, 35
	2	54.8 (d)	4.08, m	1′		35	62.8 (t)	3.93, d 4.13, d	
	3	39.4 (t)	1.52, m			36	174.2 (s)		
	4	24.8 (d)	1.66		Leu-3	NH		7.21, d (7)	
	5	22.2 (q)	0.91			38	53.6 (d)	4.39, m	39, 40, 43
	6	22.6 (q)	1.29[b]			39	41.0 (t)	1.94	
	7	175.5 (s)				40	24.8 (d)	1.63, m	
Glu	NH		9.19, d (4)			41	20.0 (q)	0.91, d	
	9	57.3 (d)	4.05, m	7, 10, 11, 13		42	20.3 (q)	0.93, d	
	10	27.0 (t)	2.12, br m			43	174.1 (s)		
	11	33.6 (t)	2.39, m	12, 9, 10	Ser-2	NH		8.28, d (8)	
	12	180.6 (s)				45	56.6 (d)	4.47, m	43, 46, 47
	13	176.1 (s)				46	61.9 (t)	3.78, d 4.05	
Thr	NH		8.49, d (6.5)			47	171.9 (s)		
	15	60.9 (d)	4.14, m	13, 16, 17	Ile-2	NH		7.18, d (9.5)	
	16	69.5 (d)	5.49, dq, (11, 6)	15, 17, 54		49	56.6 (d)	4.64, dd (10, 3)	47, 50, 51, 52, 54
	17	17.6 (q)	1.39, d (6)	15, 16		50	36.5 (d)	1.99, m	
	18	172.3 (s)				51	24.6 (t)	0.97, m	
Ile-1	NH		8.76, d (7)			52	15.2 (q)	0.95, d (6)	
	20	53.4 (d)	4.05, m			53	11.3 (q)	0.91	
	21	24.7 (d)	1.97, m			54	168.9 (s)		

[a] Multiplicities were established using the DEPT pulse sequence.

Table 4. ^{1}H and ^{13}C NMR Assignments of Pseudophomin A (**1**) in DMSO-d_6

Residue	C #	^{13}C [a] δ (ppm) (mult)	^{1}H δ (ppm), multiplicity (J in Hz)	HMBC	Residue	C #	^{13}C [a] δ (ppm) (mult)	^{1}H δ (ppm), multiplicity (J in Hz)	HMBC
C_{12}HO-acid	1′	172.9 (s)				23	15.8 (q)	0.83, d (6.5)	
	2′	44.2 (t)	2.26, d (6.5)	1′, 3′, 4′		24	11.7 (q)	0.75, t (7)	21
	3′	68.4 (d)	3.85, m			25	172.4 (s)		
	4′	37.9 (t)	1.37, m		Leu-2	NH		7.20, br s	
	5′	29.9 (t)	1.25 [b]			27	58.1 (d)	4.30, m	25, 29
	6′	25.6 (t)	1.25 [b]			28	30.9 (t)	2.26, m	
	7′	25.8 (t)	1.25 [b]			29	24.9 (d)	1.71, br m	
	8′	25.8 (t)	1.25 [b]			30	24.8 (q)	1.63	
	9′	22.9 (t)	1.25 [b]			31	23.9 (q)	0.88, d (8.5)	
	10′	14.7 (q)	0.91			32	171.0 (s)		
Leu-1	NH		7.66, d (8)		Ser-1	NH		7.77, br s	
	2	54.4 (d)	4.08, m	1′		34	59.1 (d)	4.70, m	35
	3	32.0 (t)	1.25			35	62.6 (t)	3.63, m 3.70, m	
	4	23.9 (d)	0.83			36	171.8 (s)		
	5	21.5 (q)	0.88, d (8.5)		Leu-3	NH		7.35, d (6.5)	36
	6	21.8 (q)	0.77			38	58.1 (d)	4.08, m	
	7	174.3 (s)				39	29.5 (t)	1.25	41
Glu	NH		8.53, br s			40	25.0 (d)	1.51, m	
	9	52.7 (d)	4.16, m	7, 13		41	22.8 (q)	0.91, d	
	10	27.3 (t)	1.86, m			42	23.5 (q)	0.91, d	
	11	30.9 (t)	2.31, m	13		43	174.2 (s)		
	12	174.4 (s)			Ser-2	NH		8.29, d (7)	
	13	173.8 (s)				45	57.9 (d)	4.30, m	46
Thr	NH		8.29, d (7)	13, 15, 16		46	62.2 (t)	3.74, m 3.80, m	45
	15	59.1 (d)	4.30, m			47	171.8 (s)		
	16	69.5 (d)	5.19, dq (10, 6)	15, 17, 54	Ile-2	NH		7.66, d (8)	
	17	18.1 (q)	1.19, d (6)	15, 16		49	51.9 (d)	4.30, m	
	18	171.1 (s)				50	37.2 (d)	1.90, m	
Ile-1	NH		8.06, br s			51	26.3 (t)	1.04, m 1.31, m	
	20	57.7 (d)	4.08, m	18		52	15.8 (q)	0.91, d (7)	
	21	36.6 (d)	1.63, m	20, 25		53	12.1 (q)	0.72, d (6.5)	
	22	25.6 (t)	1.37, m			54	169.8 (s)		

[a] Multiplicities were established using the DEPT pulse sequence.

Table 5. ^{1}H and ^{13}C NMR Assignments of compound **4** (R_1 = Ac) in $(CD_3)_2CO$

Residue	C #	^{13}C [a]	^{1}H	HMBC	Residue	C #	^{13}C [a]	^{1}H	HMBC
		δ (ppm) (mult)	δ (ppm) mult (*J* in Hz)				δ (ppm) (mult)	δ (ppm) mult (*J* in Hz)	
Glu	NH		8.70, br s			27	52.6 (d)	4.88, m	28, 31
	2	57.7 (d)	4.30, m	6		28	63.9 (t)	4.30, m	
	3	25.0 (t)	1.36, m			29	20.3 (q)	2.03, s	30
	4	-	2.32, dd (9.5, 6)	5		30	169.8 (s)		
	5	178.6 (s)				31	169.6 (s)		
	6	170.5 (s)			Leu-2	NH		7.12, br s	
Thr	NH		7.59, d (8.5)			33	57.2 (d)	4.46, m	31
	8	61.2 (d)	4.30, m	6, 9, 11		34	40.5 (t)	1.85, m	38
	9	70.0 (d)	5.38, dq, (12, 6)	8, 51		35	24.7 (d)	1.91	
	10	18.2 (q)	1.41, d (6)	8		36	22.5 (q)	0.99, d (6.5)	
	11	174.9 (s)				37	23.0 (q)	0.90, d	
Ile-1	NH		8.03, br s			38	172.7 (s)		
	13	61.2 (d)	3.98, t (7.5)	14, 18	Ser-2	NH		7.59, d (8.5)	
	14	35.9 (d)	2.00, m	16		40	53.2 (d)	4.66, m	41, 44
	15	25.9 (t)	1.51, m			41	63.6 (t)	4.40, m	40, 43
	16	15.3 (q)	0.96, d (6.5)			42	20.3 (q)	2.05, s	
	17	11.4 (q)	0.90, t (6.5)			43	170.3 (s)		
	18	172.7 (s)				44	169.6 (s)		
Leu-1	NH		8.10, br s		Ile-2	NH		7.74, br s	
	20	53.9 (d)	4.30, m	21, 25		46	53.4 (d)	4.30, m	44, 48, 51
	21	40.8 (t)	1.67	22, 24, 25		47	36.6 (d)	1.98	49
	22	24.8 (d)	1.91, m			48	25.8 (t)	1.20, br s	50
	23	21.0 (q)	0.98, d (6.5)			49	15.5 (q)	0.96, d (6.5)	
	24	21.9 (q)	0.95, d			50	10.8 (q)	0.90, t (6.5)	
	25	173.3 (s)				51	169.6 (s)		
Ser-1	NH		7.59, d (8.5)						

[a] Multiplicities were established using the DEPT pulse sequence.

Table 6. Culture fraction (Diaion HP20 separation) residues of strain BRG100 and 189 obtained during the period of 1999 - 2002.

Culture strain (volume)	Amount of culture fraction residues (g)											
	1	2	3	4	5	6	7	8	9	10	11	12
BRG100 (60 L[a])	49	25	8.8	3.0	0.50	0.79	0.90	0.04	0.07	0.05	0.07	0.06
189 (40 L)	223	91	9	3	0.66	0.16	0.60	0.45	0.12	0.03	0.02	0.14

[a]Culture before and after solvent extraction.

5.1.1.3 Solid medium bacterial cultures

Because the isolation of the herbicidal components contained in supernatant bacterial cultures was rather lengthy and the nutrients from the culture medium appeared to interfere or mask bioactivity assays, a solid medium was used instead. To be useful the solid medium cultures would allow the isolation of bacterial metabolites with herbicidal activity, and avoid the problems caused by components used to prepare the bacterial growth medium. Thus the bacterial strain BRG100 was cultured on TSA medium prepared in two different ratios, as described in section 4.2.1.3. Upon filtration of the methanol soluble portion from the cells, further extraction of methanol extract was carried out as described in Scheme 7. Chemical analysis of each extract was carried out by HPLC. Table 7 shows the culture and time conditions for the production of metabolites by strain BRG100 and summarizes the amount of solid bacterial cultures processed during this project (last row). HPLC analysis indicated that the components of extracts obtained from cultures on solid medium were similar to those obtained in liquid medium. In particular, both pseudophomins A and B were produced on solid medium under any set of conditions. Strain BRG100 appeared to produce the largest metabolite mass in 100 % TSA medium incubated for 96 hr.

Table 7. Extracts from strain BRG100[a] cultured on TSA[b] (solid) medium

Strain	Culture medium	Incubation period	Dry cells (amount)	MeOH extract	EtOAc extract	Aqueous extract	TSA extract
BRG100	100% TSA	48 h	336 mg	49 mg	4 mg	34 mg	913 mg
BRG100	100% TSA	72 h	329 mg	57 mg	5 mg	36 mg	3600 mg
BRG100	100% TSA	96 h	349 mg	74 mg	5 mg	51 mg	3000 mg
BRG100	50% TSA	48 h	195 mg	23 mg	NA[c]	NA[c]	NA[c]
BRG100	50% TSA	72 h	145 mg	26 mg	NA[c]	NA[c]	NA[c]
BRG100	50% TSA	96 h	166 mg	26 mg	NA[c]	NA[c]	NA[c]
BRG100	100% TSA	96 h	NA	NA	70 mg	651	NA

[a]Incubated at room temperature in darkness. [b]Trypticase soy agar. [c]NA = Not available

5.1.1.4 Ultrafiltration of culture supernatants BRG100

Ultrafiltration of culture supernatants was carried through membranes of different molecular weight cut-off (30K, 10K, 3K and 1K). Culture supernatants ultrafiltered through a membrane MWCO 10,000 (10K) gave upper and lower portions were obtained. The upper portion which contained molecules of MW > 10,000 Da was further separated through a membrane MWCO 30,000 (30K) while the first lower portion containing molecules of MW <10,000 Da, were further separated through a membrane MWCO 3,000 (3K). The first upper portion of culture supernatants ultrafiltered through a 10K membrane was diluted with water to its original volume and the ultrafiltration was repeated with 10K membrane. The upper and lower portions obtained through these membranes were bioassayed for herbicidal activity. The results of herbicidal activity of all the upper and lower portions against green foxtail are presented in Table 15-17.

5.1.2 Strain 189: fractionation, purification and chemical structure determination of new metabolites

5.1.2.1 New metabolites from supernatants

Scheme 6 was followed for the fractionation of culture supernatants of strain 189. The residues of the Diaion culture fractions were tested for herbicidal activity and analyzed by HPLC and NMR spectroscopy. The fractions showing antifungal and or herbicidal activity were further separated as described above. The most bioactive fractions yield compound A (fraction 7) and B (fraction 12); fraction 10 was analyzed y NMR and UV which indicated the presence of siderophores similar to compound A. Fraction 10 could not be further purified due to the small amount obtained.

Compound A

The HPLC analysis of culture residue fraction 7 showed one major compound at r_t 10.7 min. Further separation on a C_{18} reversed-phase silica gel column afforded compound A (24 mg), as a brown amorphous powder. The UV spectrum showed an absorption maximum at 404 nm and two shoulders at 322 nm and 265 nm (chromophore of a quinoline ring), and a maximum at 231 nm, typical of pyoverdins. The ^{1}H and ^{13}C NMR spectroscopic data of compound A was indicative of a peptide chain with a chromophore moiety (Figure 6). In the positive-ion FAB–MS spectrum, the molecular ion peak appeared at *m/z* 1270. The positive ion FAB–HRMS showed a peak at *m/z*: 1270. 5920 suggesting a molecular formula of $C_{53}H_{80}N_{19}O_{18}$ (calcd *m/z* 1270. 5928, $(M+H)^+$), consistent with both the ^{1}H and ^{13}C NMR spectra. In addition, the ^{1}H NMR spectrum showed signals likely due to NH and aromatic protons at δ_H 6.10 – 8.04. Detailed analysis of J-mod, HMQC, HMBC, and COSY spectra showed the presence of eight amino acids of which seven were identified as residues of: alanine (Ala, three), lysine (Lys, one), asparagine (Asp, one), threonine (Thr, one) and N-OH ornithine (Orn, one). The partial sequence was determined by the combination of homo and heteronuclear one and two dimensional experiments. C-H- ^{1}J - coupling were detected by HMQC, quaternary carbon atoms were identified through ^{2}J and ^{3}J - coupling. Partial sequence information was obtained by HMBC correlating amide-CO with the α-H and NH but some essential correlations between α-H and NH were not found.

Further analysis of the NMR data suggested the proposed structure shown in Figure 6. Compound A appears to represent a new type of siderophore, and we named it sideropseumin A.

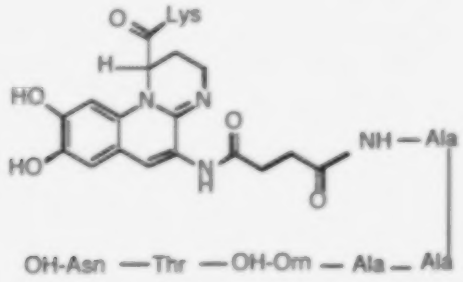


Figure 6. Proposed structure of compound A (Sideropseumin A) from strain 189

Siderophores are iron chelators produced by virtually all bacteria, fungi and some plants which serve to deliver the essential element iron into cells. Siderophores are therefore important secondary metabolites very often composed of amino acids and their derivatives. Many peptidic siderophores with diverse amino acids have been isolated from fluorescent pseudomonads; it is known that their biosynthesis occurs on non-ribosomal multi-enzyme complexes (Drechsel, Jung, 1998). The new sideropseumin A has a chromophore similar to that of pyoverdins and pseudobactins, also isolated from *Pseudomonas* species. Siderophore-binding proteins play an essential role in the uptake of iron in many Gram-pos. and Gram-neg. bacteria. Recent X-ray studies suggested that recognition of siderophores primarily occurs through interactions between the iron-hydroxamate centers of each siderophore and the side chains of several key residues in the binding pocket. Rearrangements of side chains within the binding pocket accommodate the unique structural features of each siderophore. The backbones of the siderophores are not involved in any direct interactions with the protein, demonstrating how siderophores with considerable chemical and structural diversity can be bound (Clarke et al., 2002).

Compound B — 5,6-Dihydro-2,7-dimethyl-formyl-8-(2-hydroxyethl)-pyrimido[4,5-c][1,2,6]-10H-thiadiazocine

Compound B, (7.6 mg) with a HPLC r_t 12.3 min was isolated from repeated column chromatography on a C_{18} reversed-phase silica gel column. In the MS-EI spectrum, its molecular ion peak appeared at *m/z* 280. The HRMS-EI showed a peak at *m/z*: 280.0995 suggesting a molecular formula of $C_{12}H_{16}N_4O_2S$ (calcd for 280.0993). In the ^{1}H NMR spectrum two singlets resonating at δ 2.07 and 2.41 (3H each), likely due to methyl protons, a broad singlet and a triplet at δ 2.51 and 3.51 (2H each) due to methylene protons, and two singlets at δ 7.83 and 7.88 were detected in tetradeuterated methanol). However, a signal due to an exchangeable H appeared at δ 6.72 (brs, 1H) when the ^{1}H NMR was recorded in DMSO-d_6. The multiplicities of the carbons were assigned by J-modulation. Detailed analysis of the J-mod, HMQC, HMBC, and COSY experiments showed the presence of a pyrimidine moiety. Eventually the 5,6-dihydro-2,7-dimethyl-formyl-8-(2-hydroxyethyl)-pyrimido[4,5-c][1,2,6]-10H-thiadiazocine structure was assigned for compound B (Figure 7).

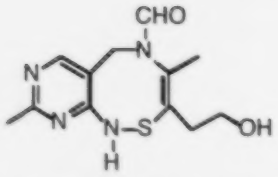
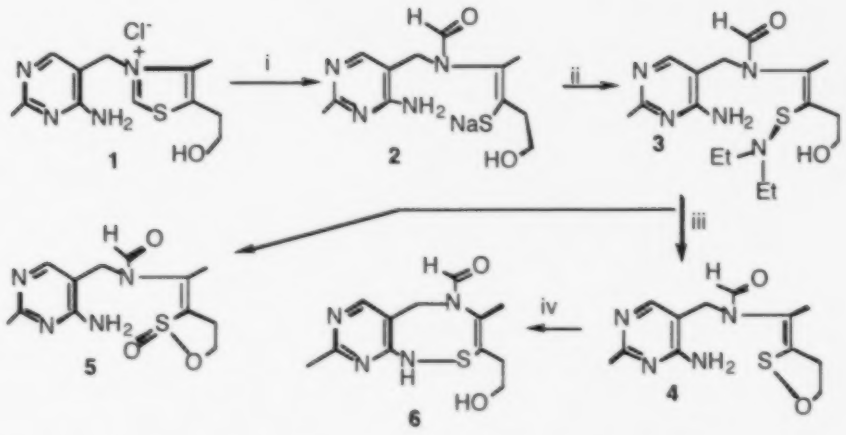


*Figure 7. Chemical structure of compound B (**6** = dihydro-2,7-dimethyl-formyl-8-(2-hydroxyethyl)-pyrimido[4,5-c][1,2,6]-10H-thiadiazocine)*

A literature search indicated that compound B had been previously isolated as a side product during chemical syntheses of thiamine analogs (Nakano 1971). Thus synthesis of compound B was carried out following this procedure. According to Scheme 9, thiamine hydrochloride was converted to its sodium salt **2** with NaOH (aq), which was oxidized in a solution of potassium iodide and iodine to yield S-amino thiamine **3**. The crude reaction mixture yielded upon fractionation the sulfinic acid ester **5** and the compound of interest pyrimido[4,5-c][1,2,6]-10H-thiadiazocine (**4**) which on standing in water at 4 °C yield 5,6-dihydro-2,7-dimethyl-formyl-8-(2-hydroxyethl)-pyrimido[4,5-c][1,2,6]-10H-thiadiazocine (**6**). Compound (**6**) is a structural isomer of (**4**), shown by their NMR and MS spectra. For example, the ^{1}H NMR spectrum of (**4**) displayed the oxymethylene signals (δ 4.12, t, 2H, J = 6.5 Hz) at higher chemical shifts than that of **6** (δ 3.52, t, 2H, J = 6.5 Hz). The spectroscopic data of compound B and **6** were identical in all respects thus confirming the structural assignment for compound B.

Compound B was also found in fractions of cultures of BRG100. It is likely that compound B was produced from thiamine hydrochloride as this was a component of the bacterial culture media. However, to the best of our knowledge, this is the first time this compound is isolated from microbial cultures; whether this compound is produced enzymatically or results from transformation of thiamine during work up/isolation of cultures could not be established. This compound was not found in non-inoculated medium, but because we never used control medium in such large amounts we believed this may be an artifact of isolation.



Scheme 9. Reagents: (i)NaOH; (ii) Et_2NH, $KI:I_2$; (iii) SiO_2; (iV) H_2O

5.1.2.2 Ultrafiltration of culture supernatants 189

Ultrafiltration of culture supernatants was carried out to separate the molecules of higher herbicidal activity from the culture supernatants through membrane of molecular weight cut-off 10K. Upper and lower portions were obtained. The results of herbicidal activity of upper and lower portions of culture supernatants 189 are presented in Table 19.

5.2 Herbicidal activity bioassays

EtOAc extracts from culture supernatants of *P. fluorescens* strain BRG100 were bioassayed against four fungal phytopathogens and the weed green foxtail. Most of the herbicidal activity remained in the aqueous phase, whereas the antifungal activity was found in the extract residue. Seeds of green foxtail were utilized to compare the herbicidal activity of extracts obtained from bacterial cultures (Scheme 2-4). Germination and root growth of green foxtail seeds was measured after 7 days of seed incubation and compared with controls (Tables 9-10). The EtOAc extracts were combined, concentrated, and fractionated by column chromatography; resulting fractions were combined and bioassayed for antifungal and herbicidal activity, employing fungal radial growth and root growth assays, as described in materials and methods. No substantial herbicidal activity was detected in any of the fractions.

Table 8. Herbicidal activity of cultures of strains BRG100 and 189 against seeds of green foxtail

	Root elongation (mm)[b]		% inhibition[c]	
Incubation medium	**BRG100**	**189**	**BRG100**	**189**
Control (glucose)[d]	13 ± 10	13 ± 10	N. I.[g]	N. I.
Culture broth supernatant (glucose)[d]	6 ± 3	2 ± 1	54	85
Culture broth supernatant (glucose)[e]	6 ± 4	5 ± 4	54	62
Control trehalose[d]	17 ± 10	17 ± 10	N. I.	N. I.
Culture broth supernatant (trehalose)[d]	6 ± 6	4 ± 3	65	76
Culture broth supernatant (trehalose)[e]	9 ± 4	12 ± 11	47	29
Control (sucrose)[d]	18 ± 7	18 ± 7	N. I.	N. I.
Culture broth supernatant (sucrose)[d]	N. A.[f]	3 ± 3	N. A.	83
Culture broth supernatant (sucrose)[e]	N. A.	6 ± 4	N. A.	67

[a] Incubated at 20^0 C in darkness. [b] Means of experiments conducted in triplicate ± standard deviation.

[c] The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(growth on bacterial culture / growth on control medium)×100].

[d] Before solvent extraction. [e] After solvent extraction. [f] Not available. [g] N. I.: no inhibition.

Table 9. Herbicidal activity of protocols A and B extracts (2 mg/mL) of cultures of strains BRG100 and 189 against seeds of green foxtail

Incubation medium	Root elongation (mm)[b]		% inhibition[c]	
	BRG100	189	BRG100	189
Protocol A				
Control[d]	12 ± 9	11 ± 8	N. I.	N. I.
Dichloromethane extract (glucose)	4 ± 4	7 ± 6	67	36
Dichloromethane extract (trehalose)	3 ± 1	4 ± 4	75	64
Ethyl acetate extract (glucose)	3 ± 2	9 ± 8	75	18
Ethyl acetate extract (trehalose)	2 ± 2	6 ± 3	83	45
Protocol B				
Control[d]	12 ± 9	12 ± 9	N. I.	N. I.
Ethyl acetate extract (glucose)	5 ± 3	5 ± 4	58	58
Ethyl acetate extract (trehalose)	4 ± 2	3 ± 3	67	75
Ethyl acetate extract (sucrose)	N. A.[f]	9 ± 7	N. A.	25
Acidic ethyl acetate extract (glucose)	4 ± 4	8 ± 5	67	33
Acidic ethyl acetate extract (trehalose)	6 ± 4	2 ± 3	50	83
Acidic ethyl acetate extract (sucrose)	N. A.	5 ± 4	N. A.	58
Protocol A				
Control[e]	11 ± 9	10 ± 7	N. I.	N. I.
Butanol extract (glucose)	5 ± 4	9 ± 4	45	10
Butanol extract (trehalose)	7 ± 6	7 ± 3	36	30
Butanol extract (sucrose)	9 ± 7	7 ± 5	18	30
Protocol B				
Control[e]	11 ± 9	10 ± 7	N. I.	N. I.
Butanol extract (glucose)	6 ± 5	12 ± 8	45	N. I.
Butanol extract (trehalose)	11 ± 7	10 ± 9	N. I.	N. I.
Butanol extract (sucrose)	N. A.	7 ± 5	N. A.	30

[a] Incubated at 20 °C in darkness. [b] Means of experiments conducted in triplicate ± standard deviation.
[c] The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(growth on extract / growth on control) × 100]. [d] Solvent: dichloromethane - methanol (1:1, v/v). [e] Solvent: dichloromethane - methanol - water (4.4:4.6:1.0, v/v). [f] N. A.: not available. [g] N. I.: no inhibition.

Table 10. Herbicidal activity[a] of ethyl acetate extract from cultures of Pseudomonas fluorescens *and pseudophomins A and B against seeds of green foxtail*

Compound (concentration)	**Root elongation (mm)**	**% inhibition[b]**
Ethyl acetate extract (0.6 mg/ mL)	10 ± 1	63 ± 6
pseudophomin A (**1**) 5×10^{-4} M	9 ± 2	67 ± 6
pseudophomin B (**2**) 5×10^{-4} M	20 ± 2	30 ± 4

[a]Incubated at 20^0 C in darkness using method C described in the experimental.

[b]The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(growth in treated / growth in control) × 100] ± standard error; results are the means of five independent experiments.

Table 11. Herbicidal activity against green foxtail of fractions obtained from culture supernatant (in glucose medium)[a] (Diaion HP20[b])

Incubation medium	Root elongation (mm)[c]		% inhibition[d]		Root elongation (mm)[c]		% inhibition[d]	
	Non-freeze-dried supernatant				Freeze-dried supernatant			
	BRG100	189	BRG100	189	BRG100	189	BRG100	189
Control	48 ± 3	36 ± 5	N.I.[e]	N.I.[e]	43 ± 3	41 ± 5	N.I.[e]	N.I.[e]
Fraction 1	0 ± 0	0 ± 0	100	100	0 ± 0	0 ± 0	100	100
Fraction 2	4 ± 1	20 ± 7	92	44	3 ± 1	4 ± 1	93	90
Fraction 3	13 ± 3	32 ± 6	73	11	11 ± 2	14 ± 3	74	66
Fraction 4	29 ± 8	29 ± 6	40	19	27 ± 6	27 ± 8	37	34
Fraction 5	31 ± 9	29 ± 5	35	19	28 ± 5	36 ± 7	35	12
Fraction 6	29 ± 7	21 ± 6	40	42	31 ± 4	33 ± 9	29	20
Fraction 7	22 ± 8	29 ± 8	54	19	17 ± 6	19 ± 7	61	54
Fraction 8	29 ± 8	31 ± 7	40	14	33 ± 7	36 ± 7	23	12
Fraction 9	26 ± 8	21 ± 5	46	42	22 ± 6	37 ± 7	48	10
Fraction 10	44 ± 6	19 ± 4	8	47	39 ± 7	20 ± 6	9	51
Fraction 11	36 ± 7	24 ± 6	25	33	28 ± 6	32 ± 7	35	22
Fraction 12	24 ± 6	24 ± 6	50	33	22 ± 6	35 ± 7	50	15

[a] Incubated at 20 °C in darkness.

[b] Fractionation of culture supernatant after extraction with ethyl acetate.

[c] Means of experiments conducted in triplicate ± standard error.

[d] The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(growth on fraction / growth on control medium) ´ 100] . [e] No inhibition.

Table 12. Herbicidal activity of fractions (Diaion HP20) from culture medium[a] against green foxtail

Incubation medium	Root elongation (mm)[b]	% inhibition[c]
Non-freeze-dried culture medium		
Control	37 ± 4	N.I.[d]
Fraction 1	2 ± 0	95
Fraction 2	33 ± 5	N.I.[d]
Fraction 3	34 ± 5	N.I.[d]
Fraction 4	34 ± 6	N.I.[d]
Fraction 5	34 ± 4	N.I.[d]
Fraction 6	37 ± 4	N.I.[d]
Fraction 7	37 ± 4	N.I.[d]
Fraction 8	28 ± 6	N.I.[d]
Fraction 9	28 ± 6	N.I.[d]
Fraction 10	37 ± 6	N.I.[d]
Fraction 11	38 ± 5	N.I.[d]
Fraction 12	33 ± 6	N.I.[d]

[a] Incubated at 20 ^{0}C in darkness. [b] Means of experiments conducted in triplicate ± standard error.

[c] The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(growth on fraction / growth on control medium) × 100] .[d] No inhibition.

Table 13. Herbicidal activity (protocol A)[a] of extracts (2 mg/mL) from cultures of strain BRG100[b] grown on 100% trypticase soy agar (TSA) against seeds of green foxtail

Incubation medium (incubation time)	Root elongation (mm)[c]	% inhibition[d]
Control	32 ± 2	N. I.[e]
Methanol extract (48 h)	25 ± 5	22
Methanol extract (72 h)	17 ± 1	47
Methanol extract (96 h)	12 ± 1	62
Control	37 ± 2	N. I.[e]
Ethyl acetate extract (48 h)	31 ± 2	16
Ethyl acetate extract (72 h)	31 ± 2	16
Ethyl acetate extract (96 h)	34 ± 2	8
Control	38 ± 1	N. I.[e]
Aqueous extract (48 h)	35 ± 1	8
Aqueous extract (72 h)	36 ± 1	5
Aqueous extract (96 h)	21 ± 2	25
Control	34 ± 2	N. I.[e]
Trypticase soy agar extract (48 h)	31 ± 2	9
Trypticase soy agar extract (72 h)	34 ± 2	N. I.[e]
Trypticase soy agar extract (96 h)	33 ± 3	3

[a]Ungerminated seeds. [b]Incubated at 20 ^{0}C in darkness. [c]Means of experiments conducted in triplicate ± standard error. [d]The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(growth on extract/ growth on control medium)*100]. [e]N. I.: no inhibition.

Table 14. Herbicidal activity (protocol B)[a] of extracts (2 mg/mL) from cultures of strain BRG100[b] grown on 100% trypticase soy agar (TSA) against seeds of green foxtail

Incubation medium (incubation time)	**Root elongation (mm)[c]**	**% inhibition[d]**
Control	24 ± 6	N. I.[e]
Methanol extract (48 h)	11 ± 4	54
Methanol extract (72 h)	6 ± 1	75
Methanol extract (96 h)	10 ± 1	58
Control	36 ± 6	N. I.[e]
Ethyl acetate extract [f](48 h)	25 ± 5	31
Ethyl acetate extract (72 h)	20 ± 5	44
Ethyl acetate extract [f](96 h)	26 ± 6	28
Control	32 ± 5	N. I.[e]
Aqueous extract (48 h)	22 ± 5	31
Aqueous extract (72 h)	22 ± 3	31
Aqueous extract (96 h)	14 ± 3	56
Control	45 ± 4	N. I.[e]
Trypticase soy agar extract (48 h)	39 ± 6	13
Trypticase soy agar extract (72 h)	37 ± 7	18
Trypticase soy agar extract (96 h)	38 ± 6	16

[a]Pre-germinated seeds. [b]Incubated at 20 ^{0}C in darkness. [c]Means of experiments conducted in triplicate ± standard error. [d]The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(growth on extract/ growth on control medium)×100]. [e]N. I.: no inhibition.

Table 15. Herbicidal activity[a] of ultra-filtered bacterial culture of P. fluorescens *strain BRG100 grown in glucose medium.*

Incubation medium	Root elongation (mm)	% inhibition[b]
Control medium	36 ± 3	N.I.
Culture	7 ± 1	80
Culture – (10K–upper)	7 ± 1	81
Culture – (10K–lower)	18 ± 2	47
Control medium	44 ± 4	N.I.
Culture	8 ± 1	82
Culture (10K– upper)→30K–(upper)	17 ± 1	61
Culture (10K–upper)→30K–(lower)	14 ± 1	68
Control medium	37 ± 2	N.I.
Culture	7 ± 1	81
Culture (10K–lower) →3K–(upper)	18 ± 2	51
Culture (10K–lower) →3K–(lower)	22 ± 1	41

[a]Incubated at 20[0] C in darkness. [b]The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(growth in treated / growth in control) × 100] ± standard error; results are the means of five independent experiments.

Table 16. Herbicidal activity[a] of ultra-filtered bacterial culture of P. fluorescens *strain BRG100 grown in glucose medium.*

Incubation medium	Root elongation (mm)	% inhibition[b]
Control medium	35 ± 2	N.I.
Culture	7 ± 1	80
Culture – (30K–upper)	6 ± 1	83
Culture – (30K–lower)	18 ± 1	50
Culture – (1K– upper)	13 ± 1	70
Culture – (1K–lower)	12± 1	72

[a]Incubated at 20[0] C in darkness. [b]The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(growth in treated / growth in control) × 100] ± standard error; results are the means of five independent experiments.

Table 17. Herbicidal activity[a] of ultra-filtered bacterial culture of P. fluorescens *strain BRG100 grown in glucose medium.*

Incubation medium	Root elongation (mm)	% inhibition[b]
Control medium	34 ± 3	N.I.
Culture	8 ± 1	76
Culture (2 × diluted)	18 ± 1	47
Diluted culture – (10K–upper)	22 ± 1	35
Diluted culture – (10K–lower)	18 ± 1	47

[a]Incubated at 20^0 C in darkness. [b]The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(growth in treated / growth in control) × 100] ± standard error; results are the means of five independent experiments.

Table 18. Herbicidal activity[a] of ultra-filtered bacterial culture of P. fluorescens *strain 189 grown in glucose medium.*

Incubation medium	Root elongation (mm)	% inhibition[b]
Control medium	43 ± 2	N.I.
Culture	13 ± 1	70
Culture – (10K–upper)	6 ± 1	86
Culture – (10K–lower)	7 ± 1	84

[a]Incubated at 20^0 C in darkness. [b]The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(growth in treated / growth in control) × 100] ± standard error; results are the means of five independent experiments.

Pseudophomins A and B showed slight inhibition (<15%) of root germination and root growth of the weed green foxtail utilizing methods B and C; however, stronger herbicidal activity was detected using method D. Interestingly pseudophomin A showed stronger inhibition (67%) of green foxtail root germination than pseudophomin B (30%) at a conc 5 × 10^{-4} M. Overall our results indicate that the antifungal activity shown by cultures of *P. fluorescens* strain BRG100 is likely due to the secretion of

pseudophomins A (**1**) and B (**2**). Furthermore, considering that strong herbicidal activity remained in the aqueous phase of extracted cultures, compounds **1** and **2** do not appear to be the main components responsible for the herbicidal activity shown by BRG100 liquid cultures. Compounds **A** and **B** isolated from strain 189 were found inactive against green foxtail.

The non-freeze-dried culture supernatants of strains BRG100 and 189 (glucose) obtained after ethyl acetate extraction were further separated on Diaion HP20 column. Herbicidal activity assays showed that the first fractions obtained from each strain caused 100% inhibition (Table 11). Similar results were observed where the nutrient medium containing glucose as carbon source was treated similarly (Table 12). These results indicated that upon several fold increase in concentration, the nutrients present in the medium have also strong herbicidal activity and this inhibition masked the actual inhibition of the bacterial metabolites. However, upon further elution, some fractions of the bacterial culture supernatants showed significant amount of herbicidal activity, which was not observed in the fractions of nutrient medium. Similar herbicidal activity was noted in the freeze-dried culture supernatants of strain BRG100 and 189, in glucose medium (Table 11). The second fraction of non-freeze-dried and freeze-dried culture supernatants of strain BRG100 showed 92% and 93% inhibition, while equivalent fraction of culture supernatants of strain 189 exhibited 44% and 90% inhibition of seed growth (green foxtail). Overall, fractions 2, 7, 9, and 12 of culture supernatant were found to be the most important fractions due to herbicidal activity. However, this herbicidal activity decreased significantly upon further fractionation.

The herbicidal activity of the methanol, ethyl acetate and aqueous extracts of bacterial cultures of strain BRG100 incubated on 100% and 50% TSA media were tested against green foxtail, ungerminated seeds (protocol B) and pre-germinated seeds (protocol C) as described in the experimental. None of the extracts obtained from 50% TSA medium showed any appreciable amount of herbicidal activity. As shown in Table 13, the methanol extract of BRG100 incubated on 100% TSA medium for 96 h showed 62% herbicidal activity at a concentration 2 mg/mL; the methanol extracts obtained at 48h and 72 h incubation time exhibited moderate activity (22% and 47% inhibition, respectively,). The ethyl acetate extracts obtained after 48, 72 and 96 h incubations showed 16%, 16% and 8% inhibition, respectively, whereas the aqueous extract of 48, 72 and 96 h incubations showed 8%, 5% and 25% inhibition.

Similar bioassays with pre-germinated seeds of green foxtail were utilized; the methanol extract of BRG100 incubated on 100% TSA medium for 48 h showed 54% herbicidal activity at a concentration 2 mg/mL. While the same type of extracts obtained at 72 and 96 h incubation time exhibited 75% and 58% inhibition respectively (Table 14). Ethyl acetate extracts of 48, 72 and 96 h incubations showed 31%, 44% and 28% inhibition, respectively, whereas the aqueous extract of 48, 72 and 96 h incubations showed 31%, 31% and 56% inhibition.

Similarly, the herbicidal activity in the upper and lower portions of ultrafiltered culture supernatants filtered through membrane MWCO 10,000 showed 81% and 47% inhibition, respectively, whereas the upper and lower portions of the first ultrafiltered upper portion through 30K showed 61% and 68% herbicidal activity. Similarly, the upper and lower portions of the first ultrafiltered lower portion through 3K membrane showed 51% and 41% inhibition, respectively (Table 15). The activity in the upper and lower portions of culture supernatants ultrafiltered through 30K membrane showed 83% and 50% inhibition. Similarly, the activity in the upper and lower portions of culture supernatants ultrafiltered through 1K membrane showed 70% and 72% inhibition respectively (Table 16). The

upper and lower portions of 2 × diluted culture supernatants showed 35% and 47% inhibition, respectively (Table 17). The herbicidal activity in the upper and lower portions of ultrafiltered culture supernatants of 189 filtered through membrane MWCO 10,000 showed 86% and 84% inhibition, respectively (Table 18).

5.3 Antifungal activity bioassays

The antifungal activity of bacterial cultures of strains BRG100, 189, and corresponding control media was tested against *P. lingam*, *A. brassicae*, *S. sclerotiorum*, and *R. solani*, as described in the experimental. The germination of fungal spores or mycelial growth in control medium was compared with that of spores or mycelium incubated in medium containing cell-free bacterial cultures combined with fungal medium, as shown in Table 19.

The bioassay results with *P. lingam* showed that the germination of fungal spores incubated in media containing cell-free bacterial cultures of strain BRG100 (glucose or trehalose media) was completely inhibited (90-100%) after 48 hr (Table 19), whereas cell-free cultures of strain 189 (glucose) were less active (48%). Furthermore, the sucrose medium did not appear to favor the production of anti-blackleg metabolites by either strain (Table 19). The bioassay results with *A. brassicae* showed that the growth of mycelia incubated in media containing cell-free bacterial cultures of either strains BRG100 or 189 (glucose or trehalose media) was somewhat inhibited (17-27%) after 6 days (Table 19), while cell-free cultures of strain 189 in sucrose (BRG100 not available) were also inhibitory (18%). The bioassay results with *S. sclerotiorum* showed that only the growth of mycelia incubated in media containing cell-free bacterial cultures of either strains BRG100 or 189 in trehalose media was slightly inhibited (12-14%) after 6 days (Table 19). The bioassay results with *R. solani* showed that the growth of mycelia incubated in media containing cell-free bacterial cultures of strain BRG100 was moderately inhibited (29%) after 6 days (Table 19).

The antifungal activity of extracts of bacterial cultures of strains BRG100 and 189 obtained according to protocols A (Table 20), and B (Table 21) and corresponding controls were tested against *P. lingam*, *A. brassicae*, *S. sclerotiorum*, and *R. solani*, as described in the experimental. The mycelial growth on control medium containing solvents utilized to dissolve extracts was compared with that of mycelium incubated on agar medium containing bacterial extracts, as shown in Tables 20 and 21. The ethyl acetate extracts (protocol A) of BRG 100 showed strong antifungal activity to both *P. lingam* (58-61%) and *A. brassicae* (54-57%) whereas the dichloromethane extract of strain 189 showed stronger activity against *S. sclerotiorum* (100%) (Table 20). The butanol extracts of both strains were inhibitory to *A. brassicae*, *S. sclerotiorum*, and *R. solani* but less inhibitory to *P. lingam*(Table 20). The ethyl acetate extracts (protocol B) of BRG100 showed strong antifungal activity to both *P. lingam* (63%) and *S. sclerotiorum* (72%) at a concentration of 2 mg/mL whereas the ethyl acetate extract of strain 189 showed stronger activity against *A. brassicae* (100%) (Table 21). The butanol extracts of both strains were more inhibitory *R. solani* than to the other fungi (Table 21). at a concentration of 2 mg/mL) and *S. sclerotiorum* (Table 21), 76% at a concentration of 2 mg/mL) and moderate activity against, *R. solani* (Table 22, 46% at a concentration of 2 mg/mL).

Table 19. Antifungal activity[a] of cell-free cultures (supernatants) of strains BRG 100 and 189

Incubation medium	*Phoma lingam*[b] % inhibition[d]		*Alternaria brassicae*[c] % inhibition[d]		*Sclerotinia sclerotiorum*[c] % inhibition[d]		*Rhizoctonia solani*[c] % inhibition[d]	
	BRG 100	**189**	**BRG 100**	**189**	**BRG 100**	**189**	**BRG 100**	**189**
Control (glucose)	N. I.	N. I.	N. I.	N. I.	N. I.	N. I.	N. I.	N. I.
Cell-free culture (glucose)	100% ± 1	48% ± 2	27% ± 1	23% ± 1	N. I.	N. I.	29% ± 3	N. I.
Control (trehalose)	N. I.[e]	N. I.	N. I.	N. I.	N. I.	N. I.	N. I.	N. I.
Cell-free culture (trehalose)	90% ± 1	100% ± 1	22% ± 1	17% ± 1	14% ± 4	12% ± 4	N. I.	N. I.
Control (sucrose)	N. A.[f]	N. A.	N. A.	N. I.	N. I.	N. I.	N. I.	N. I.
Cell-free culture (sucrose)	N. A.	N. A.	N. A.	18% ± 1	N. I.	N. I.	N. I.	N. I.

[a] Incubated at 25 °C under constant light.

[b] Concentration of spores: 1×10^5 spores/well, in minimal medium (0.5 mL / well); % germination after 48 h of incubation.

[c] Mycelia incubated on potato dextrose broth (PDB, 2 mL / well); diameter of mycelial growth after 6 days of incubation.

[d] The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(diameter of mycelial growth on bacterial culture / diameter of mycelial growth in control medium) × 100]; values represent % inhibition ± standard deviation.

[e] N. I.: no inhibition. [f] N. A.: not available.

Table 20. Antifungal activity[a] of protocol A extracts (2 mg/mL) of cultures of strain BRG 100 and 189

Incubation medium	*Phoma lingam*[b] % inhibition[d]		*Alternaria brassicae*[b] % inhibition[d]		*Sclerotinia sclerotiorum*[c] % inhibition[d]		*Rhizoctonia solani* % inhibition[d]	
	BRG 100	**189**	**BRG 100**	**189**	**BRG 100**	**189**	**BRG 100**	**189**
Control[e]	N. I.[g]	N. I.	N. I.	N. I.	N. I.	N. I.	N. I.	N. I.
Dichloromethane extract (glucose)	24% ± 5	N. I.	43% ± 4	24% ± 1	32% ± 1	N. I.	N. I.	N. I.
Dichloromethane extract (trehalose)	34% ± 4	N. I.	41% ± 8	N. I.[g]	28% ± 1	100% ± 1	N. I.	N. A.[g]
Ethyl acetate extract (glucose)	61% ± 1	N. I.	54% ± 3	27% ± 10	49% ± 1	34% ± 25	25% ± 10	13% ± 8
Ethyl acetate extract (trehalose)	58% ± 3	N. A.[h]	57% ± 2	N. A.	55% ± 1	N. A.	20% ± 1	N. A.
Control[f]	N. I.	N. I.	N. I.	N. I.	N. I.	N. I.	N. I.	N. I.
Butanol extract (glucose)	16% ± 6	N. I.	14% ± 1	11% ± 6	N. I.	N. I.	70% ± 1	N. I.
Butanol extract (trehalose)	N. I.	N. I.	41% ± 8	14% ± 4	6% ± 1	60% ± 1	25% ± 11	62% ± 8
Butanol extract (sucrose)	N. I.	N. I.	22% ± 4	22% ± 6	N. I.	N. I.	38% ± 1	28% ± 8

[a] Incubated at 25 °C under constant light. [b] Mycelia incubated on V8 agar medium. [c] Mycelia incubated on potato dextrose agar (PDA).

[d] The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(diameter of mycelial growth on extract/diameter of mycelial growth in control medium) × 100]; values represent % inhibition ± standard deviation. [e] Solvent - dichloromethane - methanol (1/1, v/v). [f] Solvent: dichloromethane - methanol - water (4.4/ /4.6/1.0, v/v). [g] N. I.: no inhibition. [h] N. A.: not available.

Table 21. Antifungal activity[a] of protocol B culture extracts (2 mg/mL) of strains BRG 100 and 189

Incubation medium	*Phoma lingam*[b] % inhibition[d]		*Alternaria brassicae*[b] % inhibition[d]		*Sclerotinia sclerotiorum*[c] % inhibition[d]		*Rhizoctonia solani*[c] % inhibition[d]	
	BRG 100	**189**	**BRG 100**	**189**	**BRG 100**	**189**	**BRG 100**	**189**
Control[e]	N. I.[g]	N. I.	N. I.	N. I.	N. I.	N. I.	N. I.	N. I.
Ethyl acetate extract (glucose)	63% ± 3	N. I.	49% ± 4	49% ± 3	68% ± 8	15% ± 1	50% ± 1	N. I.
Ethyl acetate extract (trehalose)	63% ± 1	38% ± 9	54% ± 1	100% ± 1	74% ± 1	42% ± 9	22% ± 5	48% ± 11
Ethyl acetate extract (sucrose)	N. A.[g]	30% ± 4	N. A.	57% ± 1	N. A.	N. I.	N. A.	35% ± 4
Acidic ethyl acetate extract (glucose)	16% ± 3	N. I.	35% ± 11	27% ± 11	61% ± 6	N. I.	42% ± 13	26% ± 7
Acidic ethyl acetate extract (trehalose)	24% ± 1	5% ± 1	41% ± 10	100% ± 1	61% ± 6	25% ± 4	1% ± 2	54% ± 8
Acidic ethyl acetate extract (sucrose)	N. A.	N. I.	N. A.	16% ± 1	N. A.	N. I.	N. A.	N. I.
Control[f]	N. I.	N. I.	N. I.	N. I.	N. I.	N. I.	N. I.	N. I.
Butanol extract (glucose)	16% ± 1	N. I.	24% ± 2	14% ± 6	N. I.	6% ± 7	25% ± 5	60% ± 1
Butanol extract (trehalose)	N. I.	N. I.	16% ± 6	24% ± 10	N. I.	N. I.	70% ± 6	N. I.
Butanol extract (sucrose)	N. A.	N. I.	N. A.	19% ± 1	N. A.	N. I.	N. A.	55% ± 9

[a] Incubated at 25 °C under constant light. [b] Mycelia incubated on V8 agar medium. [c] Mycelia incubated on potato dextrose agar (PDA).

[d] The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(diameter of mycelial growth on extract/diameter of mycelial growth in control medium) × 100]; values represent % inhibition ± standard deviation. [e] Solvent -

dichloromethane - methanol (1/1, v/v). [f] Solvent: dichloromethane - methanol - water (4.4/ /4.6/1.0, v/v).[g] N. I.: no inhibition. [h] N. A.: not available.

Pseudophomins **A** and **B** along with corresponding controls were tested against the plant pathogens mentioned above. Pseudophomin B showed substantial activity (Table 22, 65%, 51% and 60% at a concentration of 0.5 mg/mL, Figures 8-10) against *P. lingam*, *A. brassicae* and *S. sclerotiorum*, respectively, and 18% inhibition against *R. solani*. Pseudophomin A displayed lower antifungal activity 31%, 23% and 13%, and 19% at a concentration of 0.5 mg/mL, (Table 22). However compounds **A** and **B** isolated from strain 189 were found inactive against *P. lingam*.

Table 22. Antifungal activity[a] of ethyl acetate extract, fractions and pseudophomins A and B from cultures of strain BRG 100 grown in glucose containing medium.

Incubation medium (concentration)	***Phoma lingam* BJ 125[b]** **% inhibition[d]**	***Alternaria brassicae* AB 11[c]** **% inhibition[d]**	***Sclerotinia sclerotiorum*[c]** **% inhibition[d]**	***Rhizoctonia solani* AG 2-1[c]** **% inhibition[d]**
Control[e]	no inhibition	no inhibition	no inhibition	no inhibition
Ethyl acetate extract (2.0 mg/ml)	62% ± 2	47% ± 5	76% ± 7[f]	46% ± 15[f]
Fraction 1 (0.5 mg/ml)	70% ± 1	56 ± 3	55% ± 4[f]	43% ± 1[f]
Fraction 2 (0.5 mg/ml)	34% ± 5	24% ± 12	58% ± 1[f]	18% ± 5[f]
Pseudophomin B (0.5 mg/ml)	65% ± 3	51% ± 18	60% ± 2	18% ± 3
Pseudophomin A (0.5 mg/ml)	31% ± 2	23% ± 2	13% ± 4	19% ± 2

[a] Incubated at 25° C under constant light.
[b] Mycelia incubated on V8 agar medium.
[c] Mycelia incubated on potato dextrose agar (PDA).
[d] The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(growth in treated / growth in control) _ 100] values represent % inhibition ± standard error.
[e] Solvent - dichloromethane : methanol (1 : 1, v/v).
[f] Results of previous report

6. Conclusions

HPLC analyses of cultures of strains BRG100 and 189 indicated that different carbon sources in the culture media affected the production of metabolites by both strains quantitatively; qualitative differences did not appear significant. That is, identical constituents are produced in media containing either glucose or trehalose but these products are produced in different quantities. As expected with secondary metabolite production, quantitative variations were also observed in cultures of each strain obtained under apparently "identical" experimental conditions. The protocol for production and extraction of bioactive metabolites was optimized to yield larger amounts of bioactive extracts within a reasonable time period. Bioassays of culture supernatants indicated that strain BRG100 displayed stronger herbicidal and antifungal activity than strain 189. Furthermore, herbicidal activity tests indicated that the most active components produced by strains BRG 100 and 189 were water soluble.

Fractionation of bioactive ethyl acetate extracts obtained from strain BRG100, followed by bioassays to determine herbicidal and antifungal activity of those fractions indicated that several bioactive products were produced by strain BRG100. These purified fractions showed strong antifungal activity against important canola, rapeseed, and mustards pathogens (blackleg, blackspot, root rot, and stem rot). Importantly, our chemical analyses and derivatization of these bioactive fractions, in particular HPLC and NMR spectroscopy, allowed the structure determination of pseudophomins A and B, two major antifungal products. The complete elucidation of their chemical structures was carried out using spectroscopy and X-ray crystallography. Similar but minor pseudophomins were also characterized, but due to the extremely small amounts their biological activity was not established. However their chemical structures suggest it might be similar to those of A and B.

We established methods for larger scale separation of the bioactive components produced by bacterial strains BRG100 and 189. Fractionation of the culture supernatants of these strains showed that several fractions were significantly inhibitory against green foxtail seeds; this herbicidal activity was not observed in the fractions of nutrient control medium. However further separations and herbicidal bioassays of fractions indicated a large dilution of activity; no single component displaying herbicidal activity was established. A new siderophore, which we named sideropseumin A, was isolated from strain 189, but upon further assays no significant herbicidal activity was detected. Because these results were rather inconclusive, we decided to analyze foxtail plants and wheat inoculated with bacteria and cell free cultures, in an effort to understand the mechanism of action of bacteria and cultures constituents. However, these results have not allowed further conclusions; neither the plants (wheat or foxtail) inoculated with bacteria or with cultures displayed detectable metabolic differences from control plants.

In conclusion, we achieved all milestones set out for the duration of this project. The last milestone, "Field tests of bioactive extracts/compounds showing herbicidal activity" will be carried out at AAFC, once an appropriate delivery system is available. A few culture fractions tested for herbicidal activity are worth to test in green house/field assays.

Our overall results suggest that both bacterial strains BRG100 and 189 could be useful agents to control soil borne plant pathogens due to production of a complex "blend" of antifungal metabolites, whose structures are derived from diverse amino acids. The herbicidal components produced by these

bacterial strains appear to be a complex mixture of water soluble products with a broad range of molecular weights, extremely difficult to separate from the components of the nutrient medium. Solid medium was not a useful method to obtain potential herbicidal components. These results suggest that these bacteria have multiple mechanisms of action and that their herbicidal activity is due to multiple components secreted into the medium over a short growth period. In our hands ultrafiltration appeared to be the best method to separate herbicidal mixtures and may yield useful bioproducts upon lyofilization. However, until these products are further evaluated in appropriately formulated delivery systems their practical application remains unknown. These products were delivered and will be evaluated at AAFC by Dr. Boyetchko's group.

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8. Indications for further development

Our work suggests that both bacterial strains BRG100 and 189 could be useful agents to control soil borne plant pathogens due to production of a complex "blend" of antifungal metabolites, whose structures are derived from diverse amino acids. We suggest that evaluation of the antifungal activity of various formulations of bacterial cultures need to be carried out first in growth cabinets and if promising, in field trials.

Our work suggests that these bacteria have multiple mechanisms of action and that their herbicidal activity is likely due to multiple components secreted into the medium over a short growth period. We suggest that the most active fractions of strains BRG100 and 189 be evaluated for herbicidal activity in appropriately formulated delivery systems. If the activity is high, further analyses of these fractions should be pursued. The following products were delivered with a copy of this report to AAFC (Dr. Boyetchko's laboratory).

Cultures of strain BRG100 (600 mL)

Ultrafiltered (10K) cultures of strain BRG 100 - upper fraction (300 mL) plus lower fraction (300 mL)

Cultures of strain 189 (600 mL)

Ultrafiltered (10K) cultures of strain 189 - upper fraction (300 mL) plus lower fraction (300 mL)

Controls: ultrafiltered (10K) culture medium - upper fraction (300 mL) plus lower fraction (100 mL).

9. Information of benefit to producers, processors, and Government

Our work indicates that these bacterial strains from AAFC have multiple mechanisms of herbicidal activity; for this reason we believe that they will be extremely useful to control weeds, as weed resistance to their bioproducts will be less likely to occur.

d) Personnel

Dr. Nargis Ismail, full-time postdoctoral fellow (100% of research work committed to this project) was hired in February and took up position on March 8, 1999. The amount of $483.25 was used to pay for travel expenses from Toronto to Saskatoon of Dr. Ismail (this amount was authorized by Mr. Bruce Baumann, Program Manager). Initial salary of $2,200 per month until September 99, $2,300 per month until March 31, 2000, $2,400 per month until March 31, 2001, $2,470 per month until March, 31 2002 and $2,500 until Aug. 31 2002 (termination date).

Ms. Yanqiu Jiang, part-time technical assistant, hired in February and took up position on March and terminated in July 1999, salary of $1,100 per month.

Ms. Iwona M. Kowalczyk, a part-time technical assistant, hired in August to replace Y. Jiang terminated Dec. 1999, salary of $730 per month.

Ms. Corinne Biesenthal, a part-time technical assistant, hired in Feb 2000 terminated Oct. 2000, salary average $1,800 per month.

Ms. Vera Cecik, a part-time technical assistant, hired in Nov. 2000 terminated June 2002, salary average $1,000 per month..

e) Equipment

Equipment:

No equipment was purchased, expenses covered rental of freeze-drier, disposable supplies (including HPLC columns and supplies), chemicals and solvents, and minor glassware.

Rental of equipment:

Please note that our original proposal included in the budget the mount of $2,000 per year for 3 years, total of $6,000 for the duration of the project, to rent a rotary evaporator.

However, after initiation of this project, it became obvious that a freeze-dryer was essential to concentrate the bacterial cultures to allow an efficient extraction procedure. Upon my written request Mr. Bruce Baumann, Program Manager, authorized in September 1999 the rental of a freeze-drier au lieu of a rotary evaporator. we have paid for rental of freeze-drier $4,000.

f) Project developed materials

Optimized protocols for fractionation of bacterial cultures as described in this report.

Isolation of new bioproducts with antifungal activity as described in this report.

g) Project photos

Pictures of bioassays are attached.

h) Acknowledgements

The scientific work was carried out by Dr. Ismail, under supervision and direction of Professor Pedras and in consultation with Dr. Boyetchko.

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We also thank R. J. Anderson, Department of Chemistry, University of British Columbia, for making available to us copies of spectra of massetolide A.

We acknowledge the support, use of facilities and services, as follows:

AAFC facilities (including growth chamber and fermentation facilities, incubators, cryofreezer, laminar flow hood, etc.), and services (includes time of personnel employed by AAFC assisting in assays, computers, library services, and data analysis).

University of Saskatchewan, Department of Chemistry facilities (including NMR, UV, IR, MS spectrometers, X-ray, HPLC chromatographs, hoods, incubators, laminar flow hood, autoclaves, microscopes, etc.), and services (includes time of personnel employed by University assisting in chemical analysis, computers, and library services).

i) Expense statement

Statement will be sent directly by the University Accounting Office under separate cover.

j) ICAR data entry

This project was registered by sending an ICAR Update form to the ICAR Office at (613) 759-1419.